

REMARKS

Claims 1-18 are currently pending in the application. Claims 1-16 have been withdrawn from consideration by the Examiner as being drawn to a nonelected invention. Claim 18 has been canceled without prejudice. Claim 17 has been amended, support being found in originally filed claim 18. No new matter has been added. Claim 17 is under consideration.

Information Disclosure Statement

The office action states that neither the parent application 09/077,173 nor the instant application contain the references that have been crossed of the IDS filed September 7, 2004, and that Applicant must provide those references listed in the IDS but not found in the parent, in order for them to be considered. Applicant submits that a copy of each of these references is contained in copending application 10/811,198, ('198) as evidenced by the attached 1449 form from '198 in which the examiner considered all the references in an office action for '198 mailed on April 12, 2006.

Specification

The office action states that the nucleotide sequence disclosure does not comply with the requirements as set forth in 37 C.F.R. 1.821-1.825. Accordingly, Applicant has amended the specification so that it contains sequence tags on page 14 and with respect to Figure 1, and have provided paper and computer readable copies of the Sequence Listing required for sequence compliance. Applicants request entry of the sequence listings.

STATEMENT UNDER 37 C.F.R. §1.821 (f) and (g)

I hereby state, as required by 37 C.F.R. §1.821 (f) that the content of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 C.F.R. §1.821 (c) and (e) respectively, are the same.

I hereby state, as required by 37 C.F.R. §1.821 (g), that the enclosed submission includes no new matter.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon

Claims rejection 35 U.S.C. 101

Claims 17-18 are rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a credible, specific and substantial utility or a well established utility. Applicant respectfully traverses the rejection.

The Office Action asserts that the specification provides no guidance for the use of the claimed transgenic non-human mammals comprising a disruption in the endogenous P2Y4 receptor gene, and that the art teaches no known utility for the claimed nonhuman mammals. The Office Action further asserts that Robaye et al (2003) indicates “no physiological role of the P2Y4 receptor has yet been established and its pharmaceutical potential thus remains uncertain”.

Applicant respectfully disagrees with these assertions for the reasons stated below.

P2Y4 (SEQ ID NO:2) and Cystic Fibrosis

The specification discloses that the polypeptide of SEQ ID NO:2 is a UTP receptor, that incubation of cells expressing the polypeptide of SEQ ID NO:2 with UTP causes the accumulation of inositol tri-phosphate in Figure 4. It was well known prior to the filing date of the instant application, that UTP was considered as a therapeutic for Cystic Fibrosis, as evidenced by the University of Wisconsin’s web site (Oct 6, 2006) attached,

“Since it is known that with CF the chloride channels do not function properly, researchers have been exploring ways to activate alternative chloride channels. In 1991,

researchers administered the drug Uridine Triphosphate, or UTP, by nasal spray to people with CF and observed that the exchange of salts (sodium and chloride) and water in their nasal cells had improved.” paragraphs 1 and 2.

Further, a 1999 article by Knowles et al. (NEJM 325:533-538) teaches that extracellular UTP nucleotides are effective *in vivo* chloride secretagogues in the nasal epithelia of patients with cystic fibrosis, see abstract, attached.

Merten et al. (1998) (Eur. J. Biochem. 251:19-24) teaches that cystic fibrosis results from a mutation in a gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) that lead to a defect in cAMP stimulated chloride transport, see first paragraph on page 19. Saleh et al. (1999) (Infection and Immunity 67(10):5076-5082) teach that human tracheal gland serous cells express CFTR and are able to respond to nucleotides through nucleotide receptor P2Y₄ with an increase in chloride transport, see first full paragraph on page 5077.

P2Y₄ Knockout Mice

Robaye et al. (2003) (Molec. Pharm. 63(4):777-783)(of record) teach that the UTP and ATP induced chloride secretory responses observed in wild-type mice are abolished in P2Y₄-null mice, in their work to evaluate the pharmacotherapeutic potential of the P2Y₄ receptor in the treatment of cystic fibrosis. Robaye teaches prior to the effective filing date of the instant application, it was known that one important action of extracellular ATP and UTP is to stimulate the transepithelial secretion of chloride as a result of increased apical permeability, referencing a 1991 article by Knowles, see first paragraph of discussion. Robaye also teaches that this process is mediated by an inositol triphosphate mediated increase in cytosolic Ca²⁺ that induces the opening of outwardly rectifying chloride channels, referencing a 1994 article by Clarke et al., see first paragraph of discussion, consistent with the instantly disclosed accumulation of inositol tri-phosphate upon incubation of cells expressing the polypeptide of SEQ ID NO:2 with UTP (Figure 4 of the instant specification).

Thus, the combined teachings of these references provide a reasonable correlation between the activity in question (UTP signaling mediated through cell surface P2Y₄ (SEQ ID NO:2)), and the P2Y₄'s physiological role in cystic fibrosis. That is, the actions of extracellular nucleotides (UTP) are mediated by P2Y₄ receptors as disclosed in the specification, and that P2Y₄ receptors are a pharmacotherapeutic target for the treatment for cystic fibrosis as asserted in the specification.

Robaye et al analyzes the data obtained from the P2Y₄ knockout mice and discusses it with respect to cystic fibrosis several times throughout the article, see, for example, the discussion section. A later publication by Robaye and colleagues (Ghanem et al. (2005) Br J Pharmacol.146(3):364-9) provides additional data from P2Y₄ knockout mice, as well as data obtained from P2Y₂ knockout mice(see attached Reference) . This additional data further substantiates the potential of P2Y₄ as a target for the treatment of intestinal complications in cystic fibrosis, thus promoting the use of the P2Y₄ knock out mice as an animal model that can be used in developing cystic fibrosis therapies targeting P2Y₄. Therefore, Applicant submits there is a credible, real world use for the claimed P2Y₄ knock out mice, thus establishing utilities are both specific and substantial.

In light of these remarks, Applicant respectfully requests reconsideration and withdrawal of the rejection.

Claims rejection 35 U.S.C. 112, first paragraph, enablement

Claims 17-18 are rejected under 35 USC 112, first paragraph as failing to comply with the enablement requirement. Applicant respectfully traverses.

The office action states on page 7 that nothing in the specification provides guidance as to how an artisan would arrive at a non-human model of disease comprising a disruption of Py24 in its genome. Applicant submits that the technology of making knock-out animals, in particularly mice, as required by newly

amended claim 17, was well established at the time of the invention, and is an established means of establishing animal models of disease. The office action states that the art of transgenesis is unpredictable, and provides examples of knockout mice with no change in phenotype or unexpected phenotypes. Applicant submits that such unpredictability are isolated incidents and can be accommodated by methods known to one of skill in the art, such as by developing several different strains of knockout mice and/or by cross breeding. Further Applicant notes that P2Y4 belongs to the family of G coupled protein receptors, of which knock out animals have been successfully produced. The Office action further states that the use of the claimed knockout mouse as a model of disease or use in an application for human therapy is not clear at the time of filing. However, Applicant contends that application of these mice for human therapy is not required to be explicit, as evidenced by the following excerpt from *In re Brana* 51 F.3d at 1567, 34 USPQ2d at 1442.

“The PTO's argument that "such in vivo tests in animals are only preclinical tests to determine whether a compound is suitable for processing in the second stage of testing, by which he apparently means in vivo testing in humans, and therefore are not reasonably predictive of the success of the claimed compounds for treating cancer in humans" confused "the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption.””

The office action states that while the art teaches transgenic mice comprising the knockout construct, the specification and the art do not provide guidance for the full breadth of the claims, indicating that the art does not teach how to obtain ES cells from other species of mammals such that an artisan can obtain a line of transgenic non-human mammals comprising the knockout construct.

Applicant respectfully disagrees noting that embryonic stem cells for animals other than mice did in fact exist at the time of filing. The Court of Customs and Patent Appeals has stated that:

For all practical purposes, the board would limit appellant to claims involving the specific materials disclosed in the examples, so that a competitor seeking to avoid infringing the claims would merely have to follow the disclosure in the subsequently-issued patent to find a substitute. However, to provide effective incentives, claims must adequately protect inventors. To demand that the first to disclose shall limit his claims to what he has found will work or to materials which meet the guidelines specified for “preferred” materials in a process such as the one herein involved would not serve the constitutional purpose of promoting the useful arts.

(*In re Goffe*, 191 USPQ 429, at 431, citing *In re Fuetterer*, 138 U.S.P.Q. 217). And in *In re Vaeck* (947 F.2d 731, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991)), the Court stated that

... we do not imply that patent applicants in art areas currently denominated as ‘unpredictable’ must never be allowed generic claims encompassing more than the particular species disclosed in their specification. It is well settled that patent applicants are not required to disclose every species encompassed by their claims, even in an unpredictable art.

at 1445, citing *In re Angstadt*, 537 F.2d 498, 502-3, 190 U.S.P.Q. 214, 218 (C.C.P.A. 1976), emphasis original....

However, solely for the purposes of expediting prosecution, and without giving up the right to prosecute the newly omitted subject matter elsewhere, Applicant has amended claim 17 so that it is limited to the transgenic non human mammal of mouse.

In light of the amendments and above remarks, Applicant submits that the specification and the art provide guidance for an artisan to arrive at the claimed invention, and respectfully requests reconsideration of the rejection.

Claims rejection 35 U.S.C. 112, second paragraph

Claims 17 and 18 are rejected under 35 U.S.C. 12, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

Specifically, the office action states that there is lack of antecedent basis for the phrase “said mouse” recited in claim 17. Accordingly, Applicant has amended claim 17 to provide antecedent basis for said phrase.

Claim 18 has been canceled by Applicant, making the rejection of claim 18 moot. In light of the amendments and above remarks, Applicant respectfully requests reconsideration of the rejection

Priority

The office action asserts that Applicant’s prior-filed applications Application No. 10/753,695 and 09/077,173 (now patent US 6,790,626) fail to provide adequate support or enablement for a transgenic non-human mammal which has a phenotypic abnormality due to a disruption in a P2Y4 gene. Applicant respectfully traverses.

Applicant notes that support for claim 17 is found on page 17, lines 45-65 of Application Serial No. 09/077,173 (now patent US 6,790,626). Applicant submits that the specification and the art provide guidance for an artisan to arrive at the claimed invention, as described above, and thus are entitled to the benefit of the afore-mentioned prior-filed Application No. 10/753,695 and 09/077,173 (now patent US 6,790,626). In light of these remarks, Applicant respectfully requests the benefit of Applicant’s above referenced prior filed applications

Claims Rejections – 35 U.S.C. §102

Claims 17 and 18 are rejected under – 35 U.S.C. §102(a) as being anticipated by Robaye et al., (2003), Molecular Pharmacology, 63:777-783.

Applicant respectfully traverses the rejection on the grounds that Robaye et al. is not prior art, given that Applicant claims priority and is entitled to the benefit of Applicant’s prior-filed applications Application No. 10/753,695 and 09/077,173 (now patent US 6,790,626), for the reasons discussed supra. Because Robaye et al. is not prior art, the cited reference does not anticipate newly amended claim 17. The rejection of Claim 18 is moot, having been cancelled by Applicant without prejudice.

In light of the amendments and above remarks, Applicant respectfully requests reconsideration of the rejection

Conclusion

Applicant submits that in view of the foregoing remarks, all issues relevant to patentability raised in the Office Action have been addressed. Applicant respectfully requests the withdrawal of rejections over the claims of the present invention.

Date:

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UW Cystic Fibrosis Center

Through Research

Since 1989, when the CF gene mutation was identified on chromosome 7, research to find a cure has increased dramatically. Within one year of the CF gene mutation discovery, researchers were able to correct in the laboratory the basic CFTR defect. The following summaries represent some of the research currently underway to develop a cure for people with CF.

For further information contact a CF Center near you or visit the Research section of the Cystic Fibrosis Foundation.

Activating Alternative Chloride Channels

Since it is known that with CF the chloride channels do not function properly, researchers have been exploring ways to activate alternative chloride channels.

In 1991, researchers administered the drug Uridine Triphosphate, or UTP, by nasal spray to people with CF and observed that the exchange of salts (sodium and chloride) and water in their nasal cells had improved.

Further research is underway to examine the effectiveness of using aerosolized UTP in the lower respiratory tracts of people with CF.

Gene Therapy

Researchers believe that certain viruses may be able to enter the cells that line the respiratory system and correct the genetic material, or the DNA, that causes the chloride channel to malfunction.

Laboratory studies done in the early 1990s showed this method to be successful in correcting the chloride channel of the cells that line the nasal passages of people with CF.

Researchers are now examining different kinds of viruses to determine the most effective use of gene therapy to correct the defective chloride channel in the lungs of people with CF.

Researchers are also looking at ways to use microscopic fat globules to deliver the normal gene to the cells.

Natural Airway Defenses Against Infection

Researchers recently identified defensin, a bacteria-fighting substance in the fluid that lines the airways of people with and without CF. Laboratory studies showed that defensins exposed to high concentrations of salt lost their abilities to destroy bacteria.

Since people with CF have higher levels of salt in their airway fluids, their defensins become inactivated, resulting in an increased risk for lung infections. These findings offer researchers direction for future studies of ways to correct or improve the lung immunity of people with CF.

Novel Nutritional Therapy

Fats, or fatty acids, in the body's cell walls are important for the cell to function normally. People with CF have been found to be deficient in these fatty acids.

Docosahexaenoic acid, or DHA, is a particular fatty acid that recently has been found to correct the basic defect in the organs affected by CF. In experiments using mice with symptoms of CF, the mice improved when given DHA.

Clinical trials with people who have CF are underway to evaluate the safety and effectiveness of using DHA to treat CF.

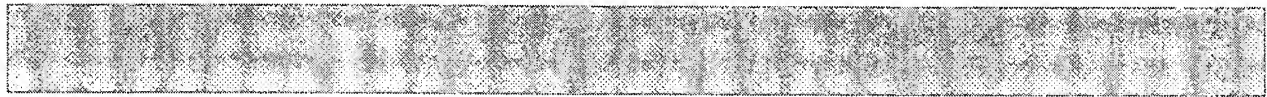
Repair Protein Therapy

Laboratory studies of 8-cyclopentyl-1, 3-dipropylxanthine, or CPX, have found that this new drug "repairs" the chloride channel defects found in the delta F508 gene mutation.

Clinical trials with people who have CF are currently underway to examine the safety and effectiveness of this drug, which can be taken orally.

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Characterization of two distinct P2Y receptors in human tracheal gland cells

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Human submucosal tracheal glands are now believed to play a major role in the physiopathology of cystic fibrosis, a genetic disease in which ATP is used as a therapeutic agent. However, actions of ATP on tracheal gland cells are poorly known. ATP-binding characteristics, and ATP-induced formation of cAMP were investigated in a cell line (MM39) of human tracheal gland cells. The binding of a radio-labelled non-hydrolysable analogue of ATP Adenosine-5'-[³⁵S]thiotriphosphate: [³⁵S]ATP[γS] was rapid (within 30 min at 4°C), stable and reversible. Scatchard analysis revealed two classes of [³⁵S]ATP[γS]-binding sites. Low-affinity binding sites had a K_{d1} of $20 \pm 5 \mu\text{M}$ ($B_{\text{max}} = 150 \text{ nmol}/10^6 \text{ cells}$) and the high-affinity binding sites had a K_{d2} of $2.5 \pm 0.2 \mu\text{M}$ ($B_{\text{max}} = 52 \text{ nmol}/10^6 \text{ cells}$). Competition experiments showed competition with ATP, ADP and 2-methylthio-ATP but no competition with UTP, AMP and adenosine. UTP stimulates protein secretion as well as it induced $[\text{Ca}^{2+}]_i$ mobilization but did not affect the intracellular cAMP levels. ATP also caused induced $[\text{Ca}^{2+}]_i$ mobilization and protein secretion but also caused an increase in cyclicAMP content of the cells, reaching a maximum after 1 min. ATP-induced cAMP formation was concentration dependent and inhibited by the P2-antagonist suramin. Reverse-transcription-PCR amplification revealed the presence of the transcripts of both the P2Y2 and the UTP-specific P2Y4 receptors. In conclusion, P2Y2 receptors, UTP-P2Y4 receptors and unidentified ATP-specific receptors seem to be present in MM39 cells which appear to be coupled differently to intracellular second-messenger systems.

Keywords: airway; purinoceptor; pyrimidinoceptor; binding; suramin.

Cystic fibrosis (CF) is a fatal hereditary disease, the most serious syndrome being mucus overabundance and persistent infection of the airways. This disease, which affects all exocrine glands is now recognized as the result of mutations in a gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR) that leads to a defect in cAMP-stimulated chloride transport [1]. Subsequently, mucus may become altered in its rheological properties and bacterial adhesivity, leading to inflammation and then mucus hypersecretion.

ATP is a potent secretagogue which binds to nucleotide P2-receptors resulting in intracellular calcium mobilization through phospholipase C (PLC) and also in PLD, PLA2 and mitogen-activated protein kinase (MAPK) activation. Several studies have shown that respiratory epithelia contain a chloride channel, distinct from CFTR, which can be activated by an increase in intracellular calcium and which is not defective in CF [2]. Therefore, nucleotides such as ATP and UTP have been proposed as therapeutic agents to bypass the altered function of CF and to restore chloride transport in CF patients [3].

P2 receptors were first classified pharmacologically, based on potency order of agonists [4]. They were subdivided in six classes. P2x are receptor-operated cationic channels selectively

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Abbreviations. ATP[γS], adenosine-5'-thiotriphosphate; 2-MeSATP, 2-methylthio-ATP; RT, reverse transcription; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; HTGS, human tracheal gland serous; SLPI, secretory leucocyte proteinase inhibitor; PLC, phospholipase C; LDH, lactate dehydrogenase; PLD, phospholipase D; PLA2, phospholipase A2; MAPK, mitogen-activated protein kinase; SV40, simian virus 40.

recognized by α , β -methylene ATP. P2Y receptors were first characterized as phospholipase-C-coupled heptahelical receptors recognized by 2-methylthio-ATP (2-MeSATP). P2t receptors are ADP-specific receptors of platelets. P2z receptors constitute non-selective membrane pores mediating the permeabilizing effects of ATP4- and are specifically recognized by 3'-O-(benzoyl)-benzoyl ATP. P2D receptors are recognized by dinucleotide polyphosphates. P2u receptors are also phospholipase-C-coupled heptahelical receptors but are recognized by ATP and UTP. Since the cloning of the P2u, P2Y, and P2x receptors, a complete reorganization of these receptors has been proposed in which P2Y and P2u receptors were respectively called P2Y1 and P2Y2 receptors [5]. Other P2Y receptors are now identified, the P2Y3 receptor [6] found in chick but not yet in human, the P2Y4 receptor specific for UTP [7, 8], and now the P2Y5, P2Y6 and P2Y7 receptors (for review, see [5]). Recently, the P2Y5 and the P2Y7 receptors first described as ATP-specific receptors [9, 10] were clearly demonstrated as not able to transduce any second-messenger signals [11, 12] and consequently are no longer considered as purinoceptors.

Recently, by using immunohistochemical and *in situ* hybridization techniques, evidence was given of a high expression of CFTR in the serous component of the tracheal glands while it is almost undetectable in the other cells of the human bronchus [13]. Consequently, human tracheal gland serous (HTGS) cells may represent an important target for the therapy of cystic fibrosis [14].

We developed techniques to culture HTGS cells [15, 16]. As *in vivo*, cultured HTGS cells become highly polarized, secrete the secretory leucocyte proteinase inhibitor (SLPI), lactoferrin, lysozyme, and high molecular-mass macromolecules, and are re-

sponsive to adrenergic, cholinergic and peptidergic agents [17]. At present, only one report has generated interest in the purinergic regulatory mechanism in cultured HTGS cell secretion [18]. We have demonstrated that both ATP and UTP generated an intracellular-calcium mobilization mainly through the PLC-Ca²⁺ pathway and that they stimulated the secretion of both SLPI and high-molecular-mass macromolecules. The receptor for these ATP actions was preliminary characterized as a P2u purinoceptor based on the potency order of agonists.

Recently, we established and characterized a simian-virus-40 (SV40)-transformed cell line of HTGS cells, the MM39 cell line [19] which has retained the secretory characteristics of the original cells. In this study, we have further examined the properties of this P2 receptor by ligand-binding experiments on MM39 cells in order to provide a molecular basis for subsequent studies concerning the mechanisms of purinergic regulation of secretion by HTGS cells. We herein demonstrate the presence of both unusual purinoceptor coupled to generation of cAMP and a pyrimidinoceptor not coupled to generation of cAMP.

MATERIALS AND METHODS

Cell culture. Culture of the SV40-transformed human tracheal gland serous cell line (MM39 cell line) was performed as previously described [19]. Cells were cultured in a Dulbecco's modified Eagle's medium (DMEM/F12) mixture supplemented with 1% of the serum substitute Ultrosor G (Bioprepa), 0.22 g/l sodium pyruvate and 8 g/l glucose. Epinephrine (2.5 µM from a 2.5 mM stock solution made in HCl M/1000 and stored at -80°C) was routinely added to the cell culture medium in order to provide optimal growth and differentiation [17]. Cells were passaged using 0.025% trypsin (GIBCO) and 0.02% EDTA. Type I collagen-coated, Falcon disposable tissue culture flasks and 24-well plates were used. In these culture conditions, MM39 cells were reported to have conserved the physiological characteristics of the genuine cells such as the presence of cytokeratin, the expression of CFTR, and a purinergic regulated secretion of SLPI [19]. At the end of all incubations with pharmacological agents, cell viability was determined by the measurement of lactate dehydrogenase (LDH) released in the incubation medium. Samples of supernatants (30 µl) were assayed for LDH activity using the Sigma lactate dehydrogenase kit, which is a spectrophotometric assay using pyruvate and NADH as substrates.

Measurements of [³⁵S]ATP[γS] binding. For saturation experiments, confluent cells in 24-well plates were washed twice for 1 h with DMEM/F12 medium. We then added, for 1 h at 4°C, increasing concentrations (0.001 µM to 100 µM) of a mixture of Adenosine-5'-[γ-thio]triphosphate (ATP[γS]; 0.1 Ci/mmol; Amersham), in a constant volume of HBS: 10 mM Hepes, 130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaSO₄ and 1 g/l glucose (300 µl/well), containing 1 mM sodium thiophosphate (which reduces non-specific binding of ATP[γS]; [20]). Supernatants were discarded and cells were rinsed three times in HBS buffer and bound radioactivity was removed after lysis of the cells with an SDS-lysis buffer (300 µl/well of HBS containing 0.1% SDS) and analysed by liquid-scintillation counting using 10 ml/well of ACS (Amersham). Non-specific binding experiments were carried out under the same conditions but with an excess of ATP (2.5 mM). *B*_{max} and *K*_d were determined by using the non-linear LIGAND program [21].

For kinetic experiments, [³⁵S]ATP[γS] (0.1 Ci/mmol) was added to the cells and the bound radioactivity was determined after different times of incubation. In dissociation experiments, ATP[γS] was equilibrated with the cells for 60 min and dis-

sociation was initiated by the addition of 2.5 mM unlabelled ATP.

For competition experiments, cells were incubated in the presence of 0.1 µM [³⁵S]ATP[γS] for 1 h exposure in the absence or the presence of the following analogues: UTP, ATP, ADP and 2-MeSATP, AMP and adenosine (1 nM to 1 mM). Non-specific binding was determined in the presence of 2.5 mM ATP.

2-MeSATP was from RBI. All other chemicals and drugs were from Sigma.

Reverse-transcription (RT)-PCR amplification. Total RNA was purified from cellular pellets of MM39 cells (approximately 10⁷ cells) following the technique of Chomczynski et al. [22]. In detecting the expression of P2Y₂ [23] and of P2Y₄ [8] mRNA transcripts, PCR amplification of mRNA (after conversion to cDNA) was performed using the Gene AMP RNA PCR kit (Perkin Elmer/Cetus). Specific amplifications were performed using as primers 5'-CTTCAACGAGGACTTCAAG-TACGTGC-3' (nucleotide positions: 323-348 of the P2Y₂ gene) and (5'-CATGTTGATGGCGTTGAGGGTGTGG-3' (nucleotide positions 1079-1103 of the P2Y₂ gene); and 5'-ATCCTGCCACCCTCACTTCTCC-3' (nucleotide positions 137-159 of the P2Y₄ gene) and 5'-AGGCGAGAAGAC-GACTGTGC-3' (nucleotide positions 822-902 of the P2Y₄ gene). 35 cycles of amplification were used with a cycle pattern as follows: denaturation 60 s at 94°C, primer annealing for 60 s at 56°C (P2Y₂) or 55°C (P2Y₄) and extension for 150 s at 72°C. An aliquot of the final amplification solution was analysed after ethidium bromide staining of a 2% agarose gel to assess the size of the amplified fragments. Another aliquot was removed and digested by the restriction enzyme *Pst*I which cuts once the two PCR products, giving two fragments of 500 bp and 281 bp for the P2Y₂-PCR product and two fragments of 408 bp and 357 bp for the P2Y₄-PCR product. The digestion products were also run into the gel.

Measurements of intracellular cAMP. cAMP was measured in ethanol extracts of cells grown on 24-well plates using a commercially available enzyme radioimmunoassay kit (Immunotech) which allows detection of cAMP in the range 0.5-50 nM with high reproducibility (accuracy ≥90%). This kit was described by the manufacturer as not reacting (cross-reactivity ≈10⁻⁶) with the other adenine nucleosides (AMP, cGMP or ATP). Results of cAMP determinations were expressed as picomoles/million cells. Extractions were carried out either after 3 min contact with increasing concentrations of ATP (10⁻⁶ M to 10⁻⁴ M) or at increasing times (0 to 7 min) after the addition of 10⁻⁴ M of ATP which was shown to be the concentration giving the maximal secretory response. 3-min contacts with agents were also carried out in order to determine whether they induce generation of cAMP: UTP, 2-MeSATP and ATP plus suramin (Bayer AG).

Single cell [Ca²⁺]_i measurements. MM39 cells were seeded at 10⁴ cells/cm² onto glass coverslips and cultured for 48 h in complete culture medium. After four 1-h washes in serum-free medium, cells were incubated for 30 min in darkness at 37°C in a 10 mM Tris/HCl buffer containing 135 mM NaCl, 5 mM KCl, 1 mM CaCl₂ and 1 µM Fluo 3/AM (from a 0.1 mM, 3% Pluronic F127 stock solution prepared in 20% dimethylsulfoxide). Changes in fluorescence were monitored at 37°C using the same incubation buffer and a fluorescence microscopy imaging system consisting of an Olympus IMT2 inverted phase contrast microscope with a ×40 objective lens and equipped for epifluorescence and photometry with a Lhesa 4015 SIT video camera as described in detail elsewhere [24]. When the baseline level of fluorescence had stabilized, 100 µM of ATP and UTP were added to the cell preparation.

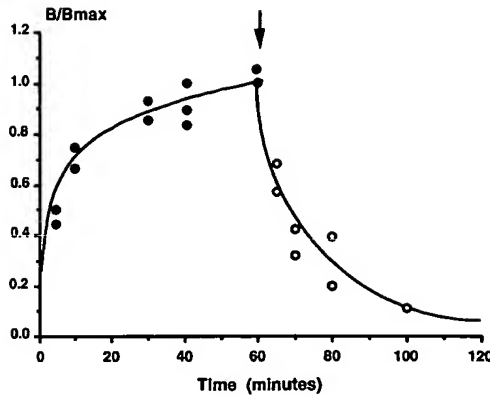


Fig. 1. Time-course for association (●) and dissociation (○) of [35 S]ATP[γ S] binding to MM39 cells. Cells (10^6 cells) were incubated at 4°C with [35 S]ATP[γ S] and binding radiolabelling was sequentially counted. The dissociation was initiated by addition of 2.5 mM ATP to the cells (arrow) and bound radioactivity was measured for various periods of time. The figure shows a representative experiment. Data are expressed as the ratio of bound [35 S]ATP[γ S] (B) to maximal [35 S]ATP[γ S] bound (B_{max}).

Pharmacological stimulation of SLPI secretion. Confluent cultures of MM39 cells grown on 24-well plates were rinsed four times for 1 h with serum-free culture medium and then exposed for 30 min to 100 μ M of nucleosides. 40 μ l of the culture medium were harvested and the SLPI was directly measured by ELISA [25]. The SLPI secretory rate was defined as the ratio of the SLPI secreted in the assays to that secreted in control experiments which were plate wells in which only vehicle solutions were added at the same times as the wells where the drugs were added. Vehicle additions were shown to be ineffective on SLPI secretion by MM39 cells. In each experiment the mean SLPI secretory rate was determined from quadruplicate assays.

Statistics. All results were expressed as means \pm SD made in quadruplicate. The significance between the effects of the concentrations of agents or between the effects of the agonists was determined by analysis of variance (ANOVA). The difference between the agents or between the concentrations of agents was isolated by the Scheffé's multiple comparison tests.

RESULTS

Binding kinetics of [35 S]ATP[γ S] by MM39 cells. All experiments were performed at 4°C in order to prevent hydrolysis of [35 S]ATP[γ S] by ectonucleotidases.

Fig. 1 shows the association and dissociation of [35 S]ATP[γ S] to MM39 cells. Association of [35 S]ATP[γ S] was rapid (steady state was achieved within 30 min at 4°C), stable and reversible. After the addition of an excess of ATP (2.5 mM), we can see that it displaced 90% of the [35 S]ATP[γ S] bound on the cells over 40 min.

The data for the saturation binding of [35 S]ATP[γ S] is shown in Fig. 2. Non-specific binding was linearly related to the radioligand concentration. It represented 25% of the total binding component at 10 μ M [35 S]ATP[γ S]. Scatchard analysis of the saturation curves obtained in four independent experiments showed that [35 S]ATP[γ S] bound to two classes of non-interacting binding sites with high ($K_{d1} = 2.5 \pm 0.2$ μ M) and low affinities ($K_{d2} = 20 \pm 5$ μ M). Corresponding B_{max} values were 35 pmol/ 10^6 cells and 400 pmol/ 10^6 cells, respectively, an 11-fold difference in the number of binding sites per cell.

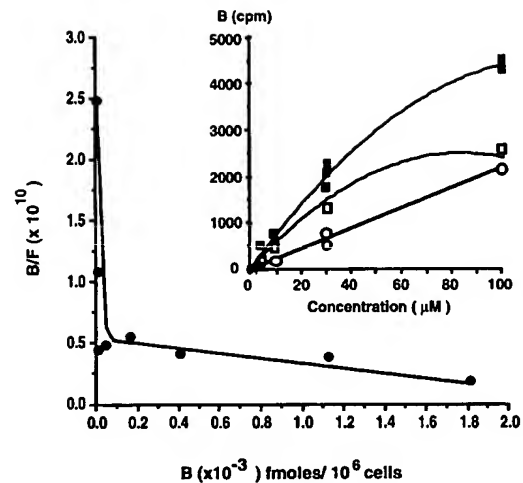


Fig. 2. Scatchard plot analysis of the dose dependence of [35 S]ATP[γ S] binding to MM39 cells. Cells (10^6 cells) were incubated with increasing concentrations (0.1 μ M to 100 μ M) of [35 S]ATP[γ S]. Non-specific binding (○) represents radioactivity in the presence of 2.5 mM ATP. Specific binding (□) refers to (total radioactivity bound; ■) – (non-specific radioactivity bound). Scatchard plot reveals a fit consistent with a two-site model. The high-affinity sites have K_{d1} values of 2.5 ± 0.2 μ M and the second low-affinity class sites has K_{d2} values of 20 ± 5 μ M).

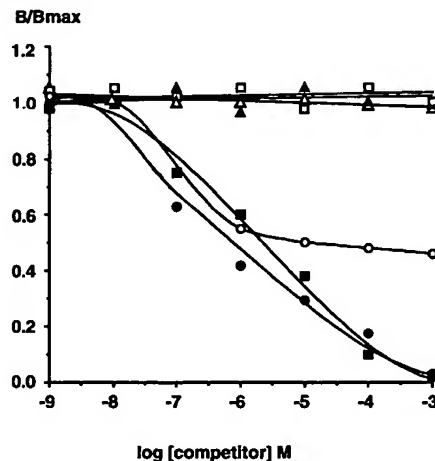


Fig. 3. Competitive displacement of 0.1 μ M [35 S]ATP[γ S] by different nucleotides. (A) Competitive binding curves for increasing concentrations of adenosine (Δ) AMP (\blacktriangle), UTP (\square), ATP (\bullet), ADP (\circ) and 2-MeSATP (\blacksquare). (B) The corresponding logit transformation of the binding curves for ATP, ADP and 2-MeSATP give IC_{50} values of 0.5 μ M, 2.3 μ M and 19 μ M, respectively. The data are representative of results from three separate experiments.

The binding specificity of [35 S]ATP[γ S] was assessed by comparing the ability of various nucleotidic analogues to inhibit [35 S]ATP[γ S] binding. Fig. 3 shows that ATP, ADP and 2-MeSATP prevented [35 S]ATP[γ S] binding to MM39 cells in a concentration-dependent manner; UTP, AMP and adenosine up to 1 mM did not. Inhibition by ADP of [35 S]ATP[γ S] binding was only partial. The IC_{50} values for concentrations of ADP needed to inhibit [35 S]ATP[γ S] binding was 2.3 μ M. In contrast, ATP and 2-MeSATP completely prevented [35 S]ATP[γ S] binding, but the inhibition curves extended over more than two orders of

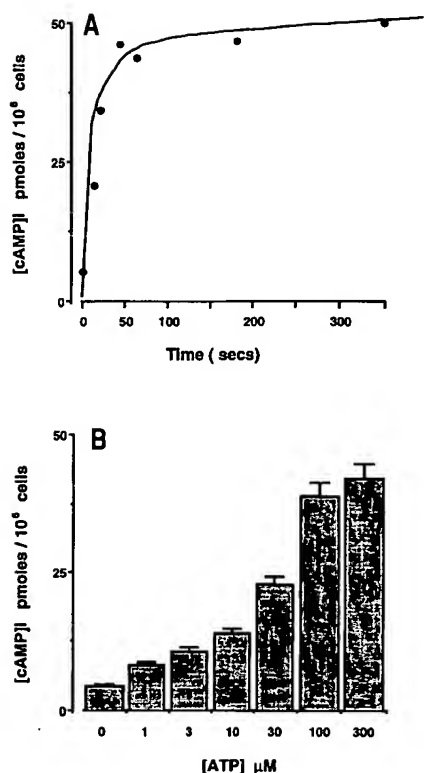


Fig. 4. Intracellular elevations of cAMP in ATP-stimulated MM39 cells. (A) Cells (10^6 cells) were stimulated with 100 μ M ATP and sequentially lysed over time. Baseline $[cAMP]_i$ values was 4 pmol/ 10^6 cells. (B) Cells (10^6 cells) were stimulated with increasing concentrations of ATP and lysed after 3 min contact with the nucleotide. Curves are representative of data obtained from three independent experiments.

magnitude of competitor concentration. This suggested that ADP recognized a simple receptor site, while ATP and 2-MeS-ATP recognized two receptor sites. This result is consistent with the Scatchard analysis.

cAMP measurements. We next examined changes in $[cAMP]_i$ levels induced by ATP. Stimulation of MM39 cells with 10^{-4} M of ATP induced $[cAMP]_i$ formation (Fig. 4A). This increase was dependent on ATP concentration (Fig. 4B). Maximum values of $[cAMP]_i$ obtained were 48 ± 2.9 pmol/ 10^6 cells, a value 10 times higher than in unstimulated cells. Concentration of ATP which gives half of the maximal $[cAMP]_i$ increase was 18 μ M.

We then investigated the ability of ATP analogues to induce elevations of $[cAMP]_i$. Neither UTP, nor 2-MeSATP used at sub-maximal concentrations (100 μ M) were able to generate any elevation of $[cAMP]_i$ in MM39 cells (Fig. 5). Only ATP was able to induce elevations in $[cAMP]_i$ which was inhibited when ATP was added to the cells with 50 μ g/ml of the P2-antagonist suramin.

Effects of UTP on MM39 cells. We next looked for the effects of UTP on biological responses by MM39 cells. Fig. 6A shows the action of nucleosides on SLPI secretion. Only ATP and UTP induced significant stimulation of SLPI secretion and to a similar extent ($+250 \pm 30\%$ and $+230 \pm 40\%$, respectively, compared with control experiment). No significant stimulation was observed with ADP, 2-MeSATP, AMP or adenosine. We also performed single cell $[Ca^{2+}]_i$ measurements which provide a

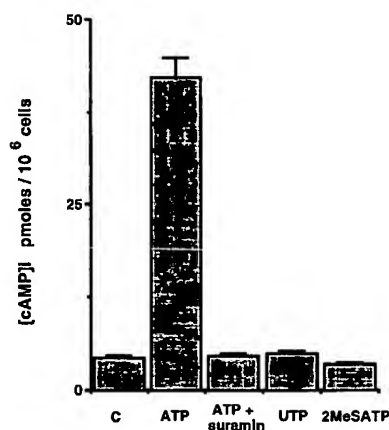


Fig. 5. Intracellular elevations of cAMP in human tracheal gland cells stimulated by different nucleotides. MM39 cells (10^6 cells) were stimulated for 3 min with 100 μ M of ATP, UTP or 2-MeSATP, or 100 μ M of ATP plus 50 μ g/ml of suramin. The figure represents means \pm SD for three independent experiments performed in duplicates.

convenient method for analysing agonist-mediated changes in $[Ca^{2+}]_i$. After the addition of 100 μ M ATP or UTP, $[Ca^{2+}]_i$ rose rapidly and then declined (Fig. 6B). The maximum $[Ca^{2+}]_i$ level reached in response to ATP or UTP were 710 ± 80 nM ($n = 14$) and 760 ± 130 nM ($n = 7$), respectively, over a baseline $[Ca^{2+}]_i$ in unstimulated MM39 cells being 120 ± 40 nM.

RT-PCR analysis of the P2Y2 and the P2Y4 receptors. We checked for the presence of the P2Y2 and P2Y4 nucleotidic receptors by PCR amplification. Electrophoresis of the PCR products revealed that MM39 cells express both the P2Y2 and the P2Y4 receptor mRNAs (Fig. 7). To verify specificity of the PCR products, digestion with the restriction enzyme *Pst*I was performed. The resulting digestion products of the amplified cDNAs migrated in the gel at the expected sizes (Fig. 7).

DISCUSSION

Tracheal glands serous cells express CFTR at high levels in the human bronchus, and represent an important target for the therapy of cystic fibrosis. Thus, it is relevant to clearly analyse and characterize the effects of therapeutic agents such as ATP and UTP (acting through P2 receptors) on these cells. We had previously given evidence for a P2u receptor on human tracheal gland serous cells based on the effects of nucleotide agonist action on intracellular calcium mobilization and on stimulation of protein secretion [18]. However in this previous work, we observed ATP-stimulated protein secretion at concentrations where intracellular calcium had not yet been mobilized. Faced with this intriguing result, the present study further characterizes the ATP-purinoreceptor by its binding to $[^3S]ATP[\gamma S]$ analyses its coupling to another second-messenger system, the adenylyl cyclase pathway.

The activation of the P2u purinoreceptor is described as being linked to increase in intracellular calcium through phospholipase C. It was also shown to be linked to inhibition of adenylyl cyclase [26] but, in some cases, also to generation of intracellular cyclicAMP ($[cAMP]_i$) in several cell types [27, 28]. Therefore, we examined the ability of ATP to induce changes in $[cAMP]_i$ levels. Extracellular ATP provoked a rapid increase in $[cAMP]_i$ by MM39 cells, which was characterized by a regular concentration/response relationship, and suggesting that the purinoreceptor

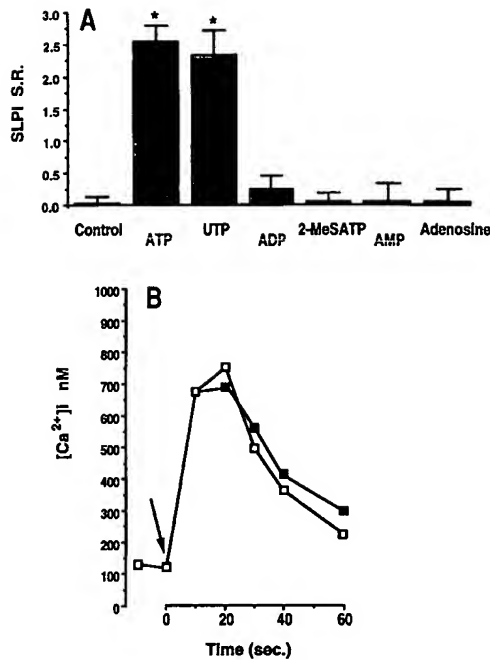


Fig. 6. Effects of UTP and ATP on MM39 cell secretion and $[Ca^{2+}]_i$ mobilization. (A) After four 1-h washes, MM39 cells were incubated for 30 min with 100 μ M ATP, UTP, 2-MeSATP or AMP. SLPI secretory rate (SR) was determined as described in Materials and Methods. Only ATP and UTP stimulated equipotently SLPI secretion. (B) $[Ca^{2+}]_i$ mobilization was measured in single cells loaded with Fluo3/AM. ATP (\square) and UTP (\blacksquare) were used at 100 μ M. Arrow, secretagogue addition to the cells. Data are from representative experiments.

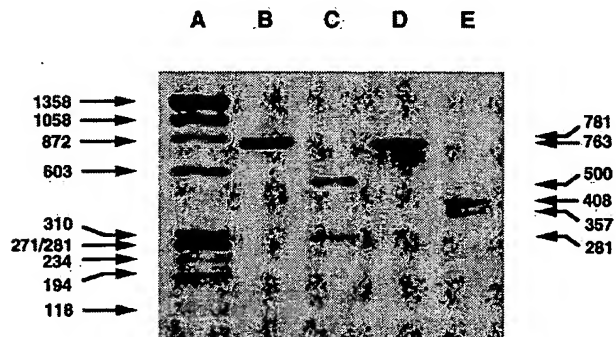


Fig. 7. RT-PCR detection of the P2Y2 and P2Y4 receptors in MM39 cells. After cDNA synthesis, samples were subjected to a 35-cycle PCR amplification. Amplification products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Lane A, molecular-size marker Φ X174/*Hae*III. Lanes B and D, P2Y2 and P2Y4 PCR products, respectively. Lane C and E, *Pst*I digestions of the P2Y2 and P2Y4 PCR products, respectively.

is positively coupled to adenylyl cyclase. Moreover, that adenosine is not a secretagogue of MM39 cells, suggesting an absence of the P1 receptor on these cells, and that the ATP-induced $[cAMP]_i$ generation is blocked by the P2-purinoreceptor antagonist suramin strongly suggest that the ATP-induced generation of $[cAMP]_i$ is caused by stimulation of the P2-purinoreceptors. This apparent coupling of the P2u receptor to adenylyl cyclase is in accordance with several other reports also describing a generation of $[cAMP]_i$ after extracellular challenge of ATP on epi-

didymal cells [29], C2C12 myotubes [30] and bovine aortic smooth muscle cells [28]. The interaction of ATP with MM39 cells has three distinguishable effects, namely stimulation of protein secretion and mobilization of $[Ca^{2+}]_i$ and activation of adenylyl cyclase. A major question currently unresolved is whether these effects of ATP are mediated by a single type of receptor separately coupled to different pathways, or whether there are different binding sites coupled to different second messenger systems. The binding of $[^3S]ATP[\gamma S]$ to the receptor did not follow simple action mass kinetics. Scatchard plots of the equilibrium saturation binding measurements were curvilinear with a Hill coefficient of less than 1, and a two-site model best fit the data. K_d s observed from either Scatchard analysis or from association and dissociation rates constants were in the micromolar range and these K_d s as well as the presence of two $[^3S]ATP[\gamma S]$ binding sites were similar to those obtained in other cell systems [30, 31]. The fact that both K_{d1} (2.5 μ M) and K_{d2} (22 μ M) have values in the same magnitude order with the previous [18], reported EC_{50} values for 1) protein secretion (1 μ M) and 2) $[Ca^{2+}]_i$ mobilization (20 μ M) and $[cAMP]_i$ elevation (18 μ M), is somewhat intriguing. It has been suggested that a rapid transition of the receptor to a desensitized state with lower affinity to ATP may occur in PC12 cells [32]. Rapid desensitization of ATP-induced cellular responses have been reported [33, 34]. It has also been proposed that ATP may stimulate different second messenger systems through distinct purinoreceptors [35]. One can suggest that the high-affinity receptor may trigger secretion and that when desensitized it induced $[Ca^{2+}]_i$ mobilization and $[cAMP]_i$ elevation in order to modulate magnitude of secretion. Although speculative, this model would explain why ATP can induce protein secretion at concentrations where no $[Ca^{2+}]_i$ mobilization and $[cAMP]_i$ elevation are yet generated (10^{-7} to 10^{-6} M) and also why this protein secretion stays maximal as soon as elevations of $[cAMP]_i$ and $[Ca^{2+}]_i$ occur (10^{-6} to 10^{-4} M). However, this obviously requires further support from studies specifically designed to test this hypothesis.

Our data also argue in favour of the presence on MM39 cells of a pyrimidoceptor specific for UTP. The P2u nucleotide receptor has been characterized as a common receptor for ATP and UTP based on identical pharmacological effects [2], coupling to G-protein activation, and cross-desensitization by ATP and UTP [36]. However, existence of a pyrimidoceptor was suggested several times [37]. With the cloning and expression of the P2Y4 subtype [7, 8], the evidence that UTP is able to interact with a UTP-specific receptor located within the cell membrane now appears unquestionable. We found that in MM39 cells, ATP and UTP effects appear to be mediated by separate and distinct receptors. This conclusion is based (a) on the presence shown by RT-PCR amplification of the mRNA of the P2Y4 subtypes, (b) on the complete lack of UTP to compete with $[^3S]ATP[\gamma S]$ on the ATP-receptor, (c) on the finding that in contrast to ATP, UTP did not activate adenylyl cyclase but (d) on the equipotency of ATP and UTP to stimulate SLPI secretion and to generate similar $[Ca^{2+}]_i$ mobilization. These results are in keeping with those of Kim et al. [31] who also showed that UTP bound poorly with ATP $[\gamma S]$ -binding sites on guinea pig tracheal epithelial cells where a P2u receptor has primarily been pharmacologically evidenced.

These results also suggest the presence of another atypical ATP-specific P2Y receptor in MM39 cells. It is likely that the P2Y2 receptor may be present since we observed the expression of the P2Y2 gene and a equipotency of UTP and ATP to induce secretion and to mobilize intracellular calcium. However, the finding that ATP and UTP did not compete for the ATP $[\gamma S]$ -binding sites and are differently coupled to adenylyl cyclase suggest the presence of another ATP-specific receptor in MM39

cells. It is unlikely that this receptor may be the P2Y₁ one because of the absence of a functional response to 2-MeSATP by the cells, the competition of 2-MeSATP for the ATP[γ S]-binding sites rather suggesting that it is a partial antagonist. No secretory responses to ADP are also likely to favour an absence of P2Y₃ receptors. The P2Y₅ and P2Y₇ receptors were first reported to be ATP-specific purinoceptors [9, 10] but it is now clearly demonstrated that these receptors can no longer be considered as purinoceptors [11, 12]. Thus, only a combination of the P2Y₄, the P2Y₂ and a yet unknown ATP-specific P2Y receptor could fit with all our results.

In summary, the present study gives evidence of the presence of both the P2Y₂ receptor and of the P2Y₄ pyrimidoceptor in HTG cells, and suggests the presence of an ATP-specific purinoceptor which appears coupled with generation of intracellular cAMP. Scatchard analyses of the binding data consistently show statistically reliable fits to a two-site binding model in which the low-affinity and the high-affinity site for ATP[γ S] differ by one order of magnitude. The low-affinity site appears to be coupled with the elevation of [cAMP]. This receptor may be specific to ATP but not for UTP. The respective physiological role of these evidenced nucleotide receptors in regulating secretion by tracheal gland cells still needs to be clearly determined.

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Pseudomonas aeruginosa Quorum-Sensing Signal Molecule *N*-(3-Oxododecanoyl)-L-Homoserine Lactone Inhibits Expression of P2Y Receptors in Cystic Fibrosis Tracheal Gland Cells

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ATP and UTP have been proposed for use as therapeutic treatment of the abnormal ion transport in the airway epithelium in cystic fibrosis (CF), the most characteristic feature of which is permanent infection by *Pseudomonas aeruginosa*. As for diverse gram-negative bacteria, this pathogenic bacterium accumulates diffusible *N*-acylhomoserine lactone (AHL) signal molecules, and when a threshold concentration is reached, virulence factor genes are activated. Human submucosal tracheal gland serous (HTGS) cells are believed to play a major role in the physiopathology of CF. Since ATP and UTP stimulate CF epithelial cells through P2Y receptors, we sought to determine whether CF HTGS cells are capable of responding to the AHLs *N*-butanoyl-L-homoserine lactone (BHL), *N*-hexanoyl-L-homoserine lactone (HHL), *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), and *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), with special reference to P2Y receptors. All AHLs inhibited ATP- and UTP-induced secretion by CF HTGS cells. The 50% inhibitory concentrations were as high as 10 and 5 μ M for BHL and HHL, respectively, but were only 0.3 and 0.4 pM for OdDHL and OHHL, respectively. Furthermore, all AHLs down-regulated the expression of the P2Y2 and P2Y4 receptors. Ibuprofen and nordihydroguaiaretic acid were able to prevent AHL inhibition of the responses to nucleotides, but neither dexamethasone nor indomethacin was able to do this. These data indicate that AHLs may alter responsiveness to ATP and UTP by CF HTGS cells and suggest that, in addition to ATP and/or UTP analogues, ibuprofen may be of use for a combinational pharmacological therapy for CF.

Cystic fibrosis (CF) is a genetic disease characterized by hypersecretion of mucus and especially by persistent severe bacterial infection and inflammation in the airways. The defect lies in mutations of a membrane protein called CF transmembrane conductance Regulator (CFTR), which possesses a cyclic AMP-dependent chloride channel activity (34) that is defective in CF. However, since calcium-dependent chloride channels remain functional in CF airway epithelial cells (46), the use of agents inducing rises in intracellular calcium was proposed for pharmacological therapy in order to bypass the basic defect in CF. Among the possible secretagogues acting on the intracellular calcium pathway, nucleotides such as ATP or UTP analogues are thought to be of most interest, as they were demonstrated to act on receptors located on the apical side of the airway epithelium (21). ATP or UTP analogues were shown to induce chloride secretion by CF epithelial cells (21) and also to induce bronchial relaxation (1). When tested clinically, nucleotides or nucleotide analogues were described as having positive and efficient effects on mucociliary clearance (3, 29).

The most characteristic feature of CF is the persistent infection of the airways by the gram-negative bacterium *Pseudomonas aeruginosa*. This bacterium is a major pathogen in CF which persists continuously (17) in the airways of CF patients

despite intense antibiotic therapy. A large number of *P. aeruginosa* virulence factors are regulated by two unique quorum-sensing systems, LasRI and RhlRI (23, 32). These two systems form a hierarchical quorum-sensing cascade (23), and there is considerable overlap within this dual-level control system in the regulation of many virulence factors. Quorum-sensing systems generally consist of two components, a small diffusible signal molecule (*N*-acylhomoserine lactone [AHL]) and a transcriptional activator protein (41). When present in sufficient amounts, the free diffusible signal molecule binds to the regulator, which activates a set of specific target genes. The threshold concentration of autoinducer necessary for the induction of the genes is reached when cultures achieve a sufficiently high bacterial density (for a review, see reference 15). Two major AHLs [*N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butanoyl-L-homoserine lactone (BHL)] and two minor AHLs [*N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) and *N*-hexanoyl-L-homoserine lactone (HHL)] have been identified in *P. aeruginosa* (47).

Since AHLs appear to readily diffuse across cell membranes (16), it is possible that these bacterial signal molecules may influence the physiology of pulmonary epithelial cells and therefore modulate the possible outcome of infection. This important aspect, that is, the capacity of bacterial AHLs to be perceived by, and to act upon, not only other bacteria but the eucaryotic host itself, has been largely ignored. Recent findings have shown that the signal molecule OdDHL could stimulate interleukin-8 (IL-8) production by respiratory epithelial cells (12) and could also influence the Th1-Th2 balance in the

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infected host (43). Despite the fact that these effects have been observed at supramaximal concentrations (10 to 30 μ M), higher than those normally encountered in laboratory media (33), these data provided us with preliminary evidence for a contribution of AHLs to the infection process.

Engelhardt et al. (14) have demonstrated that CFTR is almost undetectable in the surface epithelial cells of the human bronchus, while the serous component of the bronchotracheal glands expresses CFTR at a very high level. Human tracheal gland serous (HTGS) cells are responsible for the secretion of antibacterial and antiproteolytic proteins like lactoferrin, lysozyme, and the secretory leukocyte proteinase inhibitor (SLPI) (2). Furthermore, tracheal gland cells are able to respond to nucleotides by stimulation of SLPI secretion (26) and by an increase in chloride transport (48). The corresponding nucleotide receptors were identified as P2Y2 and P2Y4 (27). We recently developed a stable cell line of CF HTGS cells, the CF-KM4 cell line (20). In culture, these cells were shown to have retained most of their original epithelial and secretory characteristics, such as constitutive secretion of SLPI. They have also kept their CF-specific defects, such as an absence of cyclic AMP-dependent CFTR-associated chloride channel activity and an inability to respond to adrenergic and cholinergic agonists. However, like the original cells, CF-KM4 cells still respond to nucleotide stimulation. Furthermore, all of these CF-specific defects were shown to be corrected by adenovirus-mediated CFTR gene transfer.

The present work was aimed at determining the possible effects of AHLs on HTGS cells, with special reference to P2 receptors. We demonstrate here that the quorum-sensing oxo-AHLs (at up to picomolar concentrations) dramatically repressed the stimulatory effects of SLPI secretion by nucleotides, probably due to the repression of P2Y2 and P2Y4 receptor expression, in CF but not normal HTGS cells. Ibuprofen (but not glucocorticoids or indomethacin) was able to prevent this deleterious effect of OddHL on CF cells. It is therefore possible that efficiency of pharmacological CF therapy with nucleotide analogues may be hindered by minimal secretion of the quorum-sensing molecule OddHL by *P. aeruginosa*. However, we suggest that, in addition to use of ATP or UTP analogues for CF therapy, ibuprofen may be of potential use for a combinational pharmacological therapy of CF.

MATERIALS AND METHODS

Chemicals and solutions. ATP, UTP, epinephrine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), indomethacin, nortrihydroxycortic acid (NDGA), dexamethasone, cortisone, prednisone, ibuprofen, and Dulbecco's modified Eagle's medium-Ham's F12 medium were obtained from Sigma (St. Louis, Mo.). Ultraser G was from Bioprepa (Villeneuve la Garenne, France). All other chemicals were of cell culture grade.

Cell culture. The normal HTGS cell line MM39 (24) and the CF-KM4 cell line (20) were grown in a Dulbecco's modified Eagles medium-Ham's F12 medium mixture supplemented with 1% Ultraser G, 0.22 g of sodium pyruvate per liter, and 8 g of glucose per liter. Epinephrine (2.5 μ M, from a 2.5 mM stock solution made in 0.001 N HCl and stored at -80°C) was routinely added to the cell culture medium in order to provide optimal growth and differentiation. Cells were passaged by using 0.025% trypsin (GIBCO) and 0.02% EDTA. Type 1 collagen-coated Falcon disposable tissue culture flasks were used. Adenovirus-mediated CFTR gene transfer in CF-KM4 cells was carried out as previously described (20).

AHL synthesis. The general method described by Chhabra et al. (8) was used to synthesize BHL and HHL, while OddHL and OHHL were prepared as described by Dekhane et al. (11). Each compound was purified by liquid chromatography to homogeneity as determined by high-pressure liquid chromatography. The structures were confirmed by infrared as well as ^1H and ^{13}C nuclear magnetic resonance spectroscopy, and their optical purities were controlled by measurement of their optical rotations. The biological activity of each compound was tested as described previously (23).

Cytotoxicity. The cytotoxicities of the AHLs were assessed by measuring cellular dehydrogenase activity. Stock solutions of AHLs (100 μ M in dimethyl sulfoxide) were diluted in complete culture medium. For the cytotoxicity assay,

cells were grown until confluency in 24-well tissue culture plates and exposed to the different AHLs for the durations and at the concentrations indicated in Results. After incubation, dehydrogenase activity was measured biochemically by using the water-soluble tetrazolium salt MTT, which is converted to purple formazan. Results are expressed as changes in optical density compared to that of a 100% lysis control.

Pharmacological stimulation of SLPI secretion. Confluent cultures of CF-KM4 cells grown on 24-well plates were rinsed four times for 1 h each with serum-free culture medium and then exposed for 30 min to 100 μ M nucleotides. Forty microliters of the culture medium was harvested, and the SLPI was directly measured by enzyme-linked immunosorbent assay (44). The ratio of the SLPI secreted in the assays to that secreted in the assays to that secreted in control experiments was calculated, and agonist-induced stimulations are expressed as the percentage of SLPI secretion above the control value. Drugs or vehicle solutions were added at the same times to the respective wells. Vehicle additions were shown to have no effect on SLPI secretion by CF-KM4 cells. In each experiment the mean SLPI secretion rate from quadruplicate assays was determined.

Reverse transcription-PCR amplification. Total RNA was purified from cellular pellets of CF-KM4 cells (approximately 10^7 cells) by the technique of Chomczynski and Sacchi (9). Total RNA was quantified by absorbance at 260 nm (1 optical density unit = 40 mg/liter), and the quality of the preparations was monitored by agarose gel electrophoresis in the presence of formaldehyde. For detection of the expression of P2Y2 (31) and P2Y4 (10) mRNA transcripts, PCR amplification of mRNA (after conversion to cDNA) was performed with a Gene AMP RNA PCR kit (Perkin-Elmer Cetus). Specific amplifications were performed with the primers 5'-CTTCAACGAGGACTTCAAGTACGTGC-3' (nucleotides 323 to 348 of the P2Y2 gene) and 5'-CATGTTGATGGCGTTGAGG GTGTGG-3' (nucleotides 1079 to 1103 of the P2Y2 gene) for P2Y2 and 5'-ATCCTGCCACCCTCACTTCTCC-3' (nucleotides 137 to 159 of the P2Y4 gene) and 5'-AGGCGAGAAGACGACTGTGC-3' (nucleotides 882 to 902 of the P2Y4 gene) for P2Y4. Thirty-five cycles of amplification were used, with cycle times as follows: (i) denaturation for 60 s at 94°C , (ii) primer annealing for 60 s at 56°C (P2Y2) or 55°C (P2Y4), and (iii) extension for 150 s at 72°C . An aliquot of the final amplification solution was analyzed after ethidium bromide staining of a 2% agarose gel to assess the sizes of the amplified fragments. Sequences of the PCR products were checked by the dideoxy chain termination method with a Sequenase sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio).

Statistics. All results are expressed as means \pm standard deviations (SDs). The significance of differences between the effects of the concentrations of agents or between the effects of the agonists was determined by analysis of variance. The difference between agents or between concentrations of agents was isolated by the Scheffé multiple-comparison test.

RESULTS

Cytotoxicity of AHLs to CF-KM4 cells. We first checked for a possible cytotoxic effect of AHLs by using measurements of cellular dehydrogenase activity through MTT metabolism, which appeared to be a good method, since HTGS cells were shown to have a high metabolic activity (25). When added to the cells at up to 100 μ M for 24 h, neither BHL, HHL, OHHL, nor OddHL showed cytotoxicity to normal HTGS (MM39) or CF HTGS (CF-KM4) cells (data not shown).

Effects of AHLs on constitutive secretion by CF-KM4 cells. We next determined whether AHLs may alter the physiology of HTGS cells. Since these cells are glandular, we looked for protein secretion by using SLPI as a representative specific secretory marker. None of the studied AHLs were found to induce any significant change in the rate of constitutive secretion of SLPI by MM39 or CF-KM4 cells (data not shown).

Effects of AHLs on regulated secretion by CF-KM4 cells. We next looked for effects of AHLs on the ability of cells to be stimulated by secretagogues for the secretion of SLPI. MM39 cell secretion can be stimulated by either cholinergic (carbachol), adrenergic (isoproterenol), or purinergic (ATP and UTP) agonists (24, 27). A 100 μ M concentration of each secretagogue gives maximal stimulation of secretion with the following respective values above the control level: 60% \pm 12% ($P < 0.001$), 52% \pm 12% ($P < 0.001$), 65% \pm 6% ($P < 0.001$), and 60% \pm 10% ($P < 0.001$) (Fig. 1A). CF-KM4 cells do not respond to carbachol or isoproterenol but are still responsive to ATP and UTP, with stimulation of 62% \pm 6% ($P < 0.001$) and 65% \pm 10% ($P < 0.001$) above the control value, respec-

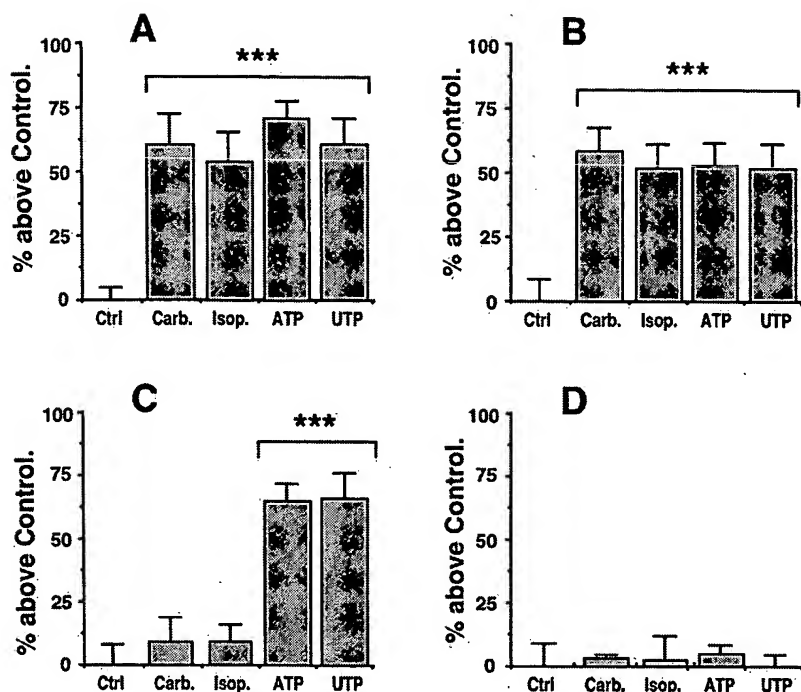


FIG. 1. Effects of OddDHL on the ability of MM39 cells (A and B) and CF-KM4 cells (C and D) to be stimulated by secretagogues. (A and C) No pretreatment; (B and D) pretreatment with OddDHL. After cells were cultured to confluency, they were washed and exposed for 30 min to the secretagogues. For each experiment, secretion of SLPI in response to 100 μ M carbachol (Carb.), isoproterenol (Isop.), ATP, and UTP are presented and expressed as the percentage of SLPI secretion above the control value (Ctrl). Each bar represents the mean and SD for quadruplicate determinations from three different experiments ($n = 12$). The statistical significance of the difference between responses to agents and the control are indicated as follows: **, $P < 0.01$; ***, $P < 0.001$. (A) MM39 cells respond to each of the secretagogues with a significant stimulation of SLPI secretion. (B) Pretreatment of the cells with 10 μ M OddDHL for 24 h does not significantly change the ability of the MM39 cells to be stimulated by any of the agonists. (C) CF-KM4 cells do not respond to carbachol and isoproterenol but still respond to ATP and UTP. (D) Pretreatment of CF-KM4 cells with 1 nM OddDHL for 24 h totally abolishes responsiveness to ATP and UTP.

tively (Fig. 1C). Pretreatment of the cells for 24 h with 10 μ M OddDHL had no significant effects on MM39 cells when they were stimulated by any of the secretagogues tested (Fig. 1B). A similar absence of effects was also observed with 10 μ M OHHL, HHL, or BHL (data not shown). However, 1 nM OddDHL led to a total loss of cell responsiveness to ATP and UTP by CF-KM4 cells (Fig. 1D). Similar results were obtained with the AHL analogue OHHL (1 nM), and with the analogues BHL and HHL but at much higher concentrations (10 μ M) (data not shown). To rule out the possibility that the differences seen between normal (MM39) and CF (CF-KM4) cells were not due to CFTR differences but to other genetic differences, we repeated the experiments with the CF-KM4 cells corrected by adenovirus-mediated gene transfer of CFTR. Under these conditions, the transfected cells showed the same phenotype as MM39 cells in response to AHLs (data not shown). This inhibition of the ability of CF-KM4 cells to be stimulated by ATP was shown to be dependent on the concentration of AHLs. Dose-response data for the action of OddDHL are presented in Fig. 2. Inhibition was shown to be total up to an OddDHL concentration of 10^{-10} M. The concentration giving half of the maximal inhibition (50% inhibitory concentration [IC_{50}]) was 0.3 pM. The calculated IC_{50} s were 0.4 pM for OHHL and 10 and 5 μ M for BHL and HHL, respectively.

Effects of AHLs on P2Y2 and P2Y4 receptor expression by CF-KM4 cells. Since the stimulatory effects of UTP and ATP were inhibited by AHLs, and especially by oxo-AHLs, at concentrations not compatible with those expected for receptor antagonists, we checked for the presence of both the P2Y2 and P2Y4 receptors by PCR amplification before and after incu-

bation of CF-KM4 cells with AHLs. We chose primers from the sequences of the cloned genes in zones with less than 40% sequence identity between the two receptors (10, 31). Electrophoresis of the PCR products revealed that CF-KM4 cells express both the P2Y2 and the P2Y4 receptor mRNAs (Fig. 3). Incubation of CF-KM4 cells with BHL (10 μ M) or OddDHL (1

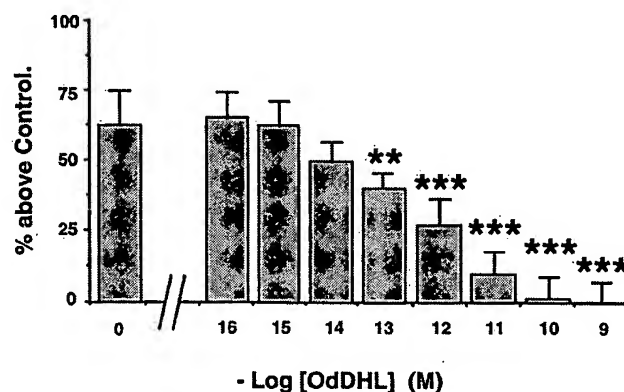


FIG. 2. Dose dependency of the inhibition by OddDHL of stimulation of SLPI secretion by ATP. After CF-KM4 cells were cultured to confluency, increasing concentrations of OddDHL were added and left for 24 h with the cells. The cells were then washed and exposed for 30 min to 100 μ M ATP. SLPI secretion was determined as described for Fig. 1. Each bar represents the mean and SD for four experiments ($n = 12$). Statistical significance is indicated as described for Fig. 1.

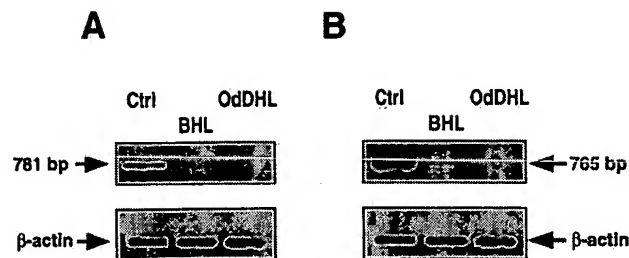


FIG. 3. Effects of OdDHL and BHL on P2Y2 (A) and P2Y4 (B) mRNA expression in CF-KM4 cells. Cells were cultured to confluency as described in the text. Cells were then exposed to 1 nM OdDHL or 10 μ M BHL for 24 h. After total RNA extraction and reverse transcription, cDNAs were amplified by using primers specific for P2Y2, P2Y4, and β -actin. The amplification products (781 bp for P2Y2 and 765 bp for P2Y4) were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Ctrl, cells without treatments.

nM) dramatically reduced (for BHL) or completely inhibited (for OdDHL) the expression of both the P2Y2 and P2Y4 receptors. This inhibition was not due to a general reduction of cell transcription, since neither of the two AHLs changed the β -actin transcript levels in CF-KM4 cells (Fig. 3).

Effects of anti-inflammatory agents. Since AHLs were shown to have immunomodulatory effects (43), we investigated whether anti-inflammatory agents might be able to modify the inhibitory effects of OdDHL (1 nM). We analyzed the effects of dexamethasone (10^{-7} M), ibuprofen (10 μ M), the cyclooxygenase inhibitor indomethacin (10 μ M), and the lipo-oxygenase inhibitor NDGA (10 μ M). These agents were added to the cells at the same time as OdDHL and left to incubate for 24 h. Figure 4 shows that neither dexamethasone nor indomethacin changed the inhibitory effects of OdDHL. Other glucocorticoids (cortisone and prednisone) gave similar results (data not shown). However, both NDGA and ibuprofen reversed the inhibitory effects of OdDHL. We observed a concentration dependence for ibuprofen prevention of the inhibitory effects of OdDHL (Fig. 5). The IC_{50} of ibuprofen was 0.8 μ M. We also assessed the effects of ibuprofen on the P2Y2 receptor mRNA by PCR amplification before and after CF-KM4 cell incubation with OdDHL (Fig. 6). Electrophoresis of the PCR products revealed that ibuprofen (10 μ M) prevented OdDHL-induced inhibition of P2Y2 receptor expression in CF-KM4 cells. In addition, as for MM39 cells, CF-KM4 cells with the defect corrected by adenovirus-mediated CFTR gene transfer expressed the P2Y2 receptor mRNA in presence of 1 nM OdDHL (Fig. 6).

DISCUSSION

The aim of the present study was to determine the effects of the quorum-sensing signal molecules AHLs secreted by *P. aeruginosa* on normal and CF HTGS cells. Normal cells respond to cholinergic, adrenergic, and purinergic agonists by an increase in protein secretion, whereas CF cells are not responsive to adrenergic and cholinergic agonists but still remain responsive to nucleotides. In the present paper we show that CF HTGS cells, but not the normal cells, are no longer able to respond to nucleotides by an increase in the secretion of SLPI in presence of minute amounts of oxo-AHLs (IC_{50} of about 0.3 pM). This absence of responsiveness is related to the repression of both the P2Y2 and P2Y4 receptor mRNAs. It is likely that this excessive sensitivity is related to the CF defect, since adenovirus-mediated CFTR gene transfer conferred resistance to oxo-AHLs that was similar to that of the normal cells.

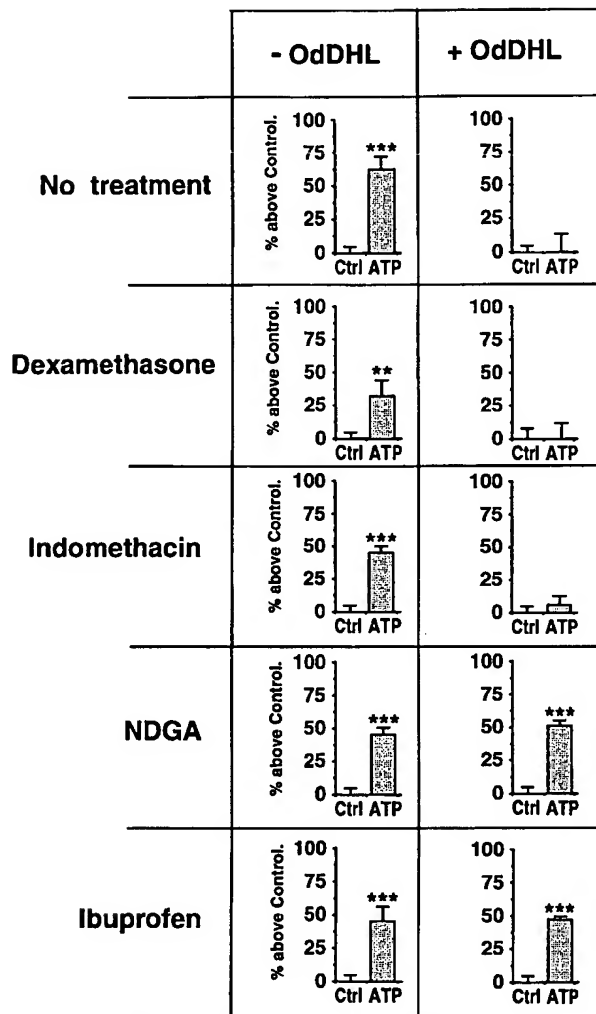


FIG. 4. Effects of immunosuppressors on OdDHL-induced inhibition of secretory responses to ATP by CF-KM4 cells. Confluent CF-KM4 cells were exposed for 24 h to 1 nM OdDHL with or without 0.1 μ M dexamethasone or 10 μ M indomethacin, NDGA, or ibuprofen. The cells were then washed and exposed for 30 min to 100 μ M ATP. The SLPI secretion was determined as described for Fig. 1. Values are given as means \pm SDs for three experiments ($n = 9$). Where no agent was added, only the vehicle was added to the cells. Statistical significance is indicated as described for Fig. 1. Note that only NDGA and ibuprofen prevented the inhibition of stimulation of SLPI secretion by ATP in CF-KM4 cells.

Another finding of this study was that ibuprofen was able to prevent the oxo-AHL-induced repression of P2Y receptors.

In CF, virulence of *P. aeruginosa* has been demonstrated to be linked to quorum-sensing density with subsequent relatively high concentrations of the autoinducers BHL and OdDHL (38). In CF lungs, *P. aeruginosa* grows to a high density, (concentrations of 10^7 to 10^8 CFU per g are common in CF sputa), at which virulence factors are expressed. This observation suggests that *P. aeruginosa* has properties which make the CF lung a good environment for bacterial development and that quorum-sensing molecules may play a role in chronic infections associated with CF. *P. aeruginosa* grows in microcolonies or in biofilms. These densities of bacteria should produce concentrations of autoinducers that would trigger the expression of specific target genes. The role of *P. aeruginosa* quorum-sensing

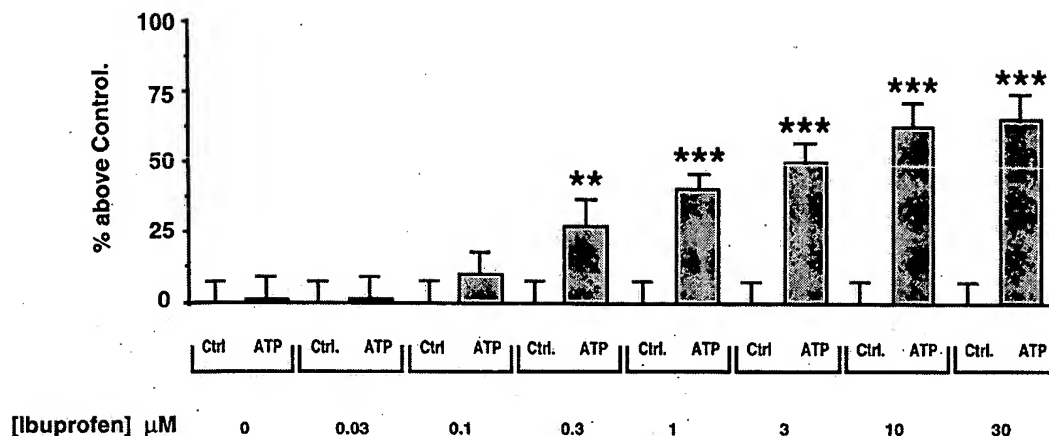


FIG. 5. Dose dependency of the prevention by ibuprofen of OdDHL inhibition of ATP-induced stimulation of SLPI secretion by CF-KM4 cells. Cells grown to confluency were exposed to 1 nM OdDHL and to increasing concentrations of ibuprofen for 24 h. The cells were then washed and exposed for 30 min to 100 μM ATP. For each concentration of ibuprofen tested, secretion of SLPI in response to 100 μM ATP is presented and compared to the control value (Ctrl). Means and SDs are shown ($n = 12$). Statistical significance is indicated as described for Fig. 1.

systems in infectious processes has not been extensively studied. It has recently been shown that *P. aeruginosa* biofilms on indwelling urethral catheters produce quorum-sensing signal molecules in situ and in vitro (37). It has also been found that a *lasR* mutant is significantly less virulent in a neonatal mouse model of infection (42) and that the *lasR* gene is expressed in the lungs of CF patients (38). Taken together, these results strongly suggest that quorum-sensing systems may play a role in infection process by regulating synthesis of several virulence factors. Nevertheless, probably because of technical difficulties, concentrations of AHLs in sputa of CF patients have never been reported. As the concentration of OdDHL normally produced in laboratory media is approximately 5 μM (33), it is reasonable to postulate that high local concentrations may be attained in sputa of CF patients, because bacteria grow as microcolonies. Since AHLs appear to readily diffuse across cell membranes, it is conceivable that these bacterial signal molecules per se may influence the outcome of the infection by modulating the host cell response. One possibility is that the quorum-sensing AHLs produced by *P. aeruginosa* could be able to modify the behavior of the CF epithelial cells which are thought to be most implicated in the pulmonary physiopathol-

ogy of the disease, i.e., the CF HTGS cells (14). This was the hypothesis that we wanted to address in this study.

There are relatively few data in the literature regarding the effects of AHLs on airway cells. The signal molecule OdDHL has been reported by some authors (12) but not by others (30) to stimulate IL-8 secretion by respiratory epithelial cells. However, this effect was observed at very high concentrations of OdDHL (over 10 μM), which are over the quorum-sensing level, and in a physiological situation where toxins and virulence factors should be secreted by the bacteria. IL-8 is a potent chemoattractant for neutrophils, whose role, when activated, is to attack, digest, and destroy bacteria at the site of infection. However, since in these reports no difference in OdDHL-induced IL-8 secretion between normal and CF cells was observed, it is unlikely (i) that this phenomenon is pathological rather than a positive reaction against infection, and (ii) that this phenomenon could be involved in the pathophysiology of CF. The reasons for this specific affinity of *P. aeruginosa* for the CF airway are still unclear. Several authors have suggested that *P. aeruginosa* may bind to some membrane receptors that are up-regulated in CF epithelial cells (7). However, these findings were not observed in vivo (45) and hardly explain the failure of the nonspecific immune system. Other authors have suggested that the up-regulation of IL-8 and down-regulation of IL-10 found in CF would generate an inflammatory state prone to *P. aeruginosa* development (5). However, this imbalance in inflammatory cytokines would, rather, protect the lung tissue and cannot explain the specificity of *P. aeruginosa*.

In contrast to the above-mentioned data, our results indicate that the quorum-sensing signal molecules OdDHL and OHHL can inhibit expression of nucleotide receptors by CF HTGS cells but not by their homologous normal, non-CF cells. This phenomenon was also observed with BHL and HHL but at very high concentrations (>10 μM), which are reached only in vitro (33). The IC_{50} s of OdDHL and OHHL are very low (0.3 and 0.4 pM, respectively), and since the secretion of oxo-AHLs by bacteria is proportional to bacterial density, these low values imply that the presence of only a small amount of bacteria is needed to lead to dramatic effects on expression of the P2Y2 and P2Y4 receptors. Very large quantities of *P. aeruginosa* in CF airways ($\geq 10^7$ CFU/g of sputum) are found during infec-

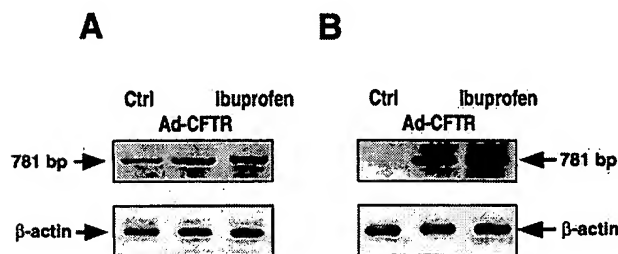


FIG. 6. Effects of OdDHL on P2Y2 mRNA expression in CF-KM4 cells with the defect corrected by adenovirus-mediated CFTR gene transfer (Ad-CFTR) or treated with ibuprofen. Cells were cultured to confluency as described in the text. Cells in absence of OdDHL (A) were compared to cells exposed to 1 nM OdDHL for 24 h (B). When used, all products (OdDHL, ibuprofen, or adenovirus) were added to the cells at the same time. After total RNA extraction and reverse transcription, cDNAs were amplified by using primers specific for P2Y2 and β -actin. The amplification products were separated by electrophoresis as described for Fig. 3.

tion, and the quantities are still significant (about 10^6 CFU/g) when infection is overcome (18). Therefore, it is possible that, due to this persistence of bacteria, the expression of P2 receptors might be continuously repressed in the airways of CF patients.

The fact that CF gland cells are normally able to respond to nucleotides by mobilization of intracellular calcium (25a), by an increase in chloride transport (48), and by stimulation of protein secretion (27) strengthens the case for the eventual use of nucleotide analogues for therapeutic purposes. ATP and UTP analogues have already been tested on healthy volunteers and on CF patients with mild CF injuries. Data about these human trials are scarce; the trials were first performed with CF patients with mild lung disease, and the data concerned chloride transport in the nose (22). Furthermore, these results were from experiments performed on epithelial tissue devoid of bacterial contamination, and no data are available for infected tissue. Currently, clinical trials are being carried out in the United States and in the United Kingdom (35). It would be very interesting to know whether and how much the benefit of the clinical treatment is impaired by infection and/or by the residual presence of bacteria.

The fact that OdDHL may induce abolition of expression of P2Y2 and P2Y4 receptors by CF-KM4 cells suggests that the action of OdDHL may lead directly or indirectly to the mobilization of inhibitory transcription factors involved in the expression of the P2Y genes. One possibility is that leukotrienes may be involved in that process, since inhibitors of leukotriene synthesis, but not inhibitors of the cyclo-oxygenase pathway, inhibited the action of oxo-AHLs. Furthermore, the high sensitivity of CF HTGS cells to oxo-AHLs, in comparison to the normal HTGS cells, suggests a CF-specific defect linked with arachidonate metabolism. It has long been known that there is an alteration of arachidonate metabolism in CF (39). However, although many investigations have detailed some cellular mechanisms involved in phospholipase A2 overactivation in CF (4, 28), neither the reasons for the overactivation (including the link with the CFTR defect) nor its physiological consequences have been determined so far. On the other hand, in contrast to ibuprofen and NDGA, glucocorticoids were shown to be inefficient in preventing OdDHL negative effects. Glucocorticoids are known to activate transcription of lipocortin-1, which is a physiological intracellular inhibitor of phospholipase A2. Glucocorticoids are thus indirectly able to inhibit generation of eicosanoids. Both NDGA, by inhibiting lipo-oxygenase, and ibuprofen, by inhibiting both cyclo-oxygenase and lipo-oxygenase, also inhibit generation of eicosanoids. Thus, the difference in the effects of (i) glucocorticoids and (ii) ibuprofen or NDGA is intriguing. A possibility is that glucocorticoids, in contrast to lipo-oxygenase inhibitors, do not function normally in CF cells. Defective inhibition of leukotriene production by leukocytes in patients with CF was reported (36), resistance of CF B lymphocytes to dexamethasone was observed (13), and inefficiency of dexamethasone in inhibiting lipopolysaccharide-induced cytokine production by CF HTGS cells was shown (19). Another possibility is that ibuprofen and NDGA may act through mechanisms other than inhibition of leukotriene production. Ibuprofen was shown to stabilize the NF- κ B-I κ B complex in the cytoplasm (40). NF- κ B is a transcription factor which is located in the cytoplasm in an inactive form, where it is bound to a protein called inhibitor- κ B α (I κ B α). Upon stimulation, I κ B α is phosphorylated and degraded, resulting in the release and translocation of NF- κ B into the nucleus, where it activates the expression of numerous genes. Ibuprofen was shown to be able to block the degradation of I κ B α and thereby to inhibit activation and translocation

of NF- κ B into the nucleus (40). NDGA was also shown to inhibit I κ B degradation (6). Further understanding of the molecular mechanism of ibuprofen action should provide insights into the process of oxo-AHL action and, more generally, into the emergence of infection and inflammation in CF patients. Here we have not been able to assess how oxo-AHLs have this particular action on CF-KM4 cells, but the consequences of this observation might be relevant. Ibuprofen has been used with success in decreasing inflammation associated with CF overinfection. That the negative effect of oxo-AHLs on P2Y expression was abolished by ibuprofen suggests a possible beneficial effect of ATP and/or UTP analogues in association with ibuprofen for CF therapy.

In conclusion, the results presented here show that oxo-AHLs from *P. aeruginosa* induce the repression of P2Y2 and P2Y4 receptors by CF-KM4 cells. It is our understanding that this work represents the first demonstration of an effect of AHLs on a secretory mechanism involved in the lung defense system in CF HTGS cells but not in normal, non-CF HTGS cells. The ability of ibuprofen to prevent this deleterious effect suggests that a possible use of ibuprofen in combination with ATP and/or UTP analogues would be of considerable interest for pharmacological therapy of CF.

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The role of epithelial P2Y₂ and P2Y₄ receptors in the regulation of intestinal chloride secretion

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1 UTP-induced chloride secretion by the intestinal mucosa mounted in Ussing chambers was assessed by measurement of the short-circuit current (I_{sc}) in the presence of phloridzin in the case of jejunum or amiloride in the case of colon to eliminate any contribution of electrogenic Na⁺ movement to the net ionic transport. Since we have previously demonstrated the absence of chloride-secretory response to apical UTP in the jejunum from P2Y₄-null mice, in the present study we studied the response to basolateral UTP in the jejunum and to either apical or basolateral UTP in the colon, in both P2Y₂- and P2Y₄-deficient mice.

2 In the jejunum, the chloride-secretory response to basolateral UTP was partially reduced in both P2Y₂- (40%) and P2Y₄- (60%) null mice.

3 In the colon, both apical or basolateral UTP increased the I_{sc} . That response was abolished in a chloride-free medium.

4 The colonic chloride-secretory response to either basolateral or apical UTP was abolished in P2Y₄-deficient mice, but not significantly affected in P2Y₂-deficient mice. The chloride-secretory response to forskolin was potentiated by prior basolateral addition of UTP and this potentiation was abolished in P2Y₄-null mice.

5 The jejunum of mice homozygous for the $\Delta F508$ mutation of cystic fibrosis transmembrane conductance regulator was responsive to UTP, but the magnitude of that response was smaller than in the wild-type littermates.

6 In conclusion, the P2Y₄ receptor fully mediates the chloride-secretory response to UTP in both small and large intestines, except at the basolateral side of the jejunum, where both P2Y₂ and P2Y₄ receptors are involved.

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; I_{sc} , short-circuit current (expressed as $\mu A cm^{-2}$); PCR, polymerase chain reaction

Introduction

It is known that ATP and UTP modulate epithelial electrolyte transport in small and large intestines. Indeed, effects on Cl[−] secretion, K⁺ secretion and Na⁺ reabsorption have been demonstrated either in mouse, rat or guinea-pig tissues or in cultured human cells (Caco-2, T84), mostly using Ussing chambers (Inoue *et al.*, 1997; Leipziger *et al.*, 1997; Kerstan *et al.*, 1998; Cressman *et al.*, 1999; McAlroy *et al.*, 2000; Lazarowski *et al.*, 2001; Smitham & Barrett, 2001; Kunzelmann & Mall, 2002; Yamamoto & Suzuki, 2002; Köttgen *et al.*, 2003; Leipziger, 2003). As bacterial invasion of epithelia induces the release of nucleotides, this response may play a role in host defense and infectious diarrhea (McNamara *et al.*, 2001; Crane *et al.*, 2002; Van Nhieu *et al.*, 2003). Although the

response to ATP is partially mediated by its degradation into adenosine and the activation of adenosine receptors (Bucheimer & Linden, 2004), the action of UTP can be explained only by the activation of P2Y receptors. Pharmacological responses to UTP can be mediated by either P2Y₂ or P2Y₄ receptors, and no selective antagonist is available to discriminate between them (Wildman *et al.*, 2003). In the airway epithelium (nasal epithelial cells, trachea), the responses to UTP ([Ca²⁺]_i increase, chloride secretion) were abolished in P2Y₂^{−/−} mice (Cressman *et al.*, 1999; Homolya *et al.*, 1999). However, the chloride-secretory response to apical UTP was maintained in the jejunum of P2Y₂-null mice. We recently generated P2Y₄-null mice in which that response was abolished (Robaye *et al.*, 2003). Extracellular nucleotide signaling in epithelia is a complex process and distinct receptors can be involved in different tissues, but also in the apical *versus* basolateral membrane (Dubyak, 2003). In the present study, we have

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systematically investigated the respective roles of P2Y₂ and P2Y₄ receptors in the apical *versus* basolateral response to UTP in both jejunum and colon.

Methods

Mice

P2Y₄-null mice have been generated in the mixed CD1-129SV genetic background, as described (Robaye *et al.*, 2003). P2Y₂^{-/-} mice initially in the B6D2 background (Homolya *et al.*, 1999), generously given by Dr BH Koller, were outbred in the 129SV background (Matos *et al.*, 2005). Mice homozygous for the ΔF508 mutation of cystic fibrosis transmembrane conductance regulator (CFTR) in the 129/FVB background (van Doornink *et al.*, 1995) were a generous gift of Dr BJ Scholte. Genotyping was performed by PCR on DNA extracted from clipped tails and using the following primers:

P2Y₂: triple primer PCR

forward primer 5'–3'	reverse primer 5'–3'	Allele
GTCACGCGCACCC	TCGGGTGCACTGCC	Wild type
TCTACTA	TTTCTT	
GGGGAACCTTCCTG	TCGGGTGCACTGC	Mutated
ACTAGG	CTTCTT	

P2Y₄: two independent PCR

forward primer 5'–3'	reverse primer 5'–3'	Allele
AGTAGAGGTTCCA	GACTCCTTGCTAT	Wild type
GTAGAAA	TCACA	
CGAAGTTATATTAA	TAATCGGTCACC	Mutated
GGGTTC	CTCA	

The genotyping of ΔF508 mice (CF mice) was performed at 21 days of age using Taqman quantitative PCR multiplex analysis of tail clip DNA. For each type of experiment, littermates were used as controls. In the case of P2Y₄, an X-linked gene, only males were used. P2Y₂^{-/-} and P2Y₄^{0/-} mice and their littermates were maintained on a standard diet in conventional facilities. ΔF508 mice and their littermates were weaned to a liquid diet (Peptamen®, Nestlé Clinical Nutrition, France) and the colony was maintained in a pathogen-free status. Mice were kept in a 12-h light–dark cycle. The studies were approved by the local Ethics Committee of Animal Welfare and conformed to the Guide for the Care and Use of Laboratory Animals adopted and promulgated by the US National Institutes of Health.

Ussing chambers measurements

Briefly, mice aged 2–5 months were killed by intraperitoneal pentobarbital (10 mg kg⁻¹). The midportion of the jejunum, extending 10 cm after the ligament of Treitz, or the distal 2 cm portion of the colon were dissected, opened and washed with Krebs bicarbonate solution. The mucosa was stripped from the adjacent muscularis layer and sealed on the basolateral side to a fixation ring with an opening diameter of 3 mm. This ring was placed between the halves of an Ussing chamber. KCl

electrodes, connected to the solution via a short agar bridge, were used for measuring the potential difference and passing current. Impedance analysis was used to determine the resistance of the epithelium and bathing solution between the voltage electrodes as described recently (Ghanem *et al.*, 2005). In this analysis, the epithelium can be represented by a lumped model consisting of a parallel circuit of a capacitance and resistance in series with the solution resistance between the voltage electrodes (R_{sol}). The resistance shunting the capacitance represents the transepithelial resistance (R_{epi}). The impedance analysis provides an evaluation of R_{sol} and $R_{sol} + R_{epi}$. In this series of experiments, the mean values were: $R_{sol} = 25 \pm 3 \text{ Ohm cm}^2$ ($n = 29$), $R_{epi} = 14 \pm 2 \text{ Ohm cm}^2$ ($n = 12$) for jejunal epithelium and $R_{epi} = 46 \pm 6 \text{ Ohm cm}^2$ ($n = 17$) for colonic epithelium. Since R_{sol} attenuates the current recorded by the voltage clamp ($I_{sc m}$), the actual short-circuit current ($I_{sc id}$) expected for an ideal voltage clamp across the epithelium was calculated as: $I_{sc id} = I_{sc m} (R_{epi} + R_{sol})/R_{epi}$. These $I_{sc id}$ values are equivalent to the net ionic flow through the epithelium and are subsequently abbreviated as I_{sc} .

The volume of each compartment bathing the jejunal or colonic mucosa was 2 ml and Krebs bicarbonate solution pre-equilibrated with a gas mixture of 5% CO₂–95% O₂ at 37°C was flowing in each compartment at a rate of 20 ml min⁻¹. The composition of the Krebs bicarbonate solution was the following in mM: Na: 140; K: 5.2; Mg: 1.2; Ca: 1.2; Cl: 120; PO₄: 2.8; HCO₃: 25; glucose: 11.5; pH 7.4. In some experiments, chloride was isoosmotically replaced by gluconate.

Study of Cl⁻-secretory response in jejunum and colon

The I_{sc} can be divided into two components: (1) a sodium absorptive component linked to the apical sodium–glucose cotransporter of villi enterocytes in the jejunum or to the apical epithelial sodium channel (ENaC) in surface colonocytes of the large intestine and (2) a chloride-secretory component linked to the existence of apical chloride channels in crypt cells of both jejunum and colon. The first component can be eliminated by addition of 1 mM phloridzin or 100 μM amiloride to the apical bath in the jejunum or colon, respectively. The small magnitude of the amiloride effect in our colon experiments is likely to be explained by the limited number of ENaC in non-sodium-restricted mice. The second component of the I_{sc} was quantitatively accounted by chloride secretion as it was abolished in chloride-free solutions in both jejunum and colon. The manœuvres of stripping the mucosa from its adjacent muscularis and its mounting in a small Ussing chamber induce the release of prostaglandins, a potential stimulus to chloride secretion that could mask other stimuli, and therefore chloride secretion was assessed as the I_{sc} following addition of phloridzin or amiloride to the apical side as well as of indomethacin (100 μM) to both bathing media. UTP was added, at 100 μM, to the apical or basolateral solution, unless otherwise stated. Forskolin was always added to the basolateral solution at the concentration of 10 μM in ethanol 0.1%; this concentration of ethanol does not affect I_{sc} (data not shown). The increase in I_{sc} (expressed in μA cm⁻²) was calculated as the difference between the basal current and the peak current obtained within 15 min of addition of UTP or forskolin. Data are expressed as mean ± s.e.m. Statistical

analysis was performed using the unpaired *t*-test and a *P*-value <0.05 was considered significant.

Materials

UTP, indomethacin, forskolin, phloridzin and amiloride were purchased from Sigma (Merelbeke, Belgium).

Results

Chloride secretion by jejunal mucosa

In order to examine the effect of UTP on intestinal chloride secretion, pieces of jejunal mucosa, stripped of muscle layers, were placed in Ussing chambers and pre-exposed to phloridzin (1 mM) and indomethacin (100 μ M). Although in these conditions no true steady state was reached within the time of study, a slow 'predictable' drift was usually obtained so that acute change in I_{sc} elicited by a given agent could be unquestionably ascribed to this stimulatory agent. Addition of UTP (100 μ M) to the basolateral side of mice jejuna increased I_{sc} (Figure 1). The magnitude of that response was significantly different in control mice from different genetic background: $69 \pm 6 \mu\text{A cm}^{-2}$ in CD1 mice and $27 \pm 3 \mu\text{A cm}^{-2}$ in 129SV mice (mean \pm s.e.m. of five animals). Within the same strain, the P2Y₄^{0/+} mice, the response to basolateral UTP was greater than the response to apical UTP, showing a mean value of $69 \pm 6 \mu\text{A cm}^{-2}$ as compared to the $17 \pm 5 \mu\text{A cm}^{-2}$ value previously reported for the apical response (Robaye *et al.*, 2003). The effect of basolateral UTP was decreased, but not completely abolished, in P2Y₄^{0/-} mice (Figure 1): $30 \pm 7 \mu\text{A cm}^{-2}$ (mean \pm s.e.m. of five animals), or a 57% inhibition. That response was also decreased in P2Y₂^{0/-} mice as compared to their control littermates: $17 \pm 2 \mu\text{A cm}^{-2}$ (mean \pm s.e.m. of five animals), or a 37% decrease. We

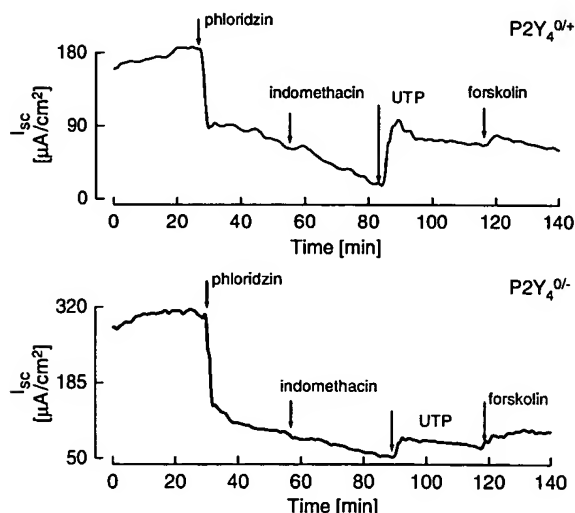


Figure 1 Stimulation of I_{sc} by basolateral UTP in the jejunum of control (upper panel) and P2Y₄-null (lower panel) mice. Sodium-glucose cotransport activity was inhibited by adding 1 mM phloridzin to the apical solution. Indomethacin (100 μ M) was added to both sides. UTP (100 μ M) and forskolin (10 μ M) were added to the basolateral bath.

confirmed that the response to apical UTP was maintained in P2Y₂^{0/-} mice, as reported previously (Cressman *et al.*, 1999) (data not shown).

Chloride secretion by colonic mucosa

In the distal colon of P2Y₄^{0/+} mice, apical and basolateral UTP increased the I_{sc} with a magnitude about two-fold smaller than that in the jejunum (Figure 2, Table 1). The responses to both apical and basolateral UTP were abolished in the colon of P2Y₄^{0/-} mice (Figure 2, Table 1). In P2Y₂^{0/-} mice, these UTP-mediated responses were not significantly different from those of their control littermates (data not shown). The response to basolateral forskolin after apical addition of UTP was not different in P2Y₄^{0/-} and control mice (Table 1). On the contrary, pre-exposure to basolateral UTP amplified the

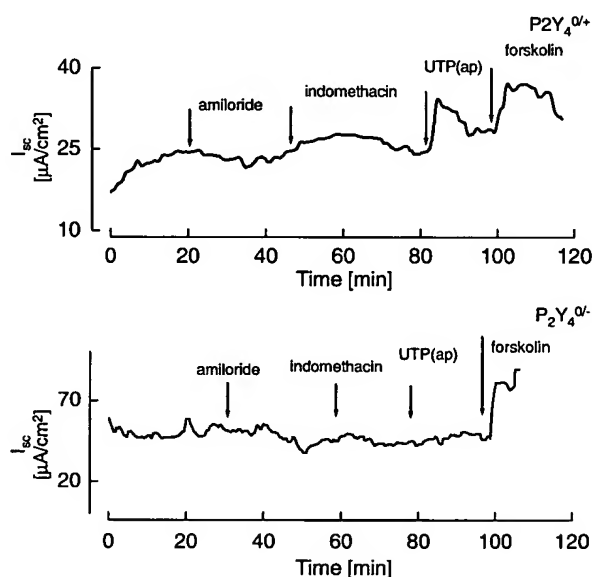


Figure 2 Stimulation of I_{sc} by apical UTP in the colon of control (upper panel) and P2Y₄-null (lower panel) mice. Sodium absorption was inhibited by adding amiloride (100 μ M) to the apical solution. The small magnitude of the amiloride effect is probably related to the limited number of ENaC in non-sodium-restricted mice. Indomethacin (100 μ M) was added to both sides. UTP (100 μ M) was added to the apical bath and forskolin (10 μ M) to the basolateral one.

Table 1 Increase of the I_{sc} in mouse colon stimulated by UTP: comparison between control and P2Y₄-deficient mice

	P2Y ₄ ^{0/+}	P2Y ₄ ^{0/-}	<i>N</i> (<i>P</i>)
UTP (apical)	11 \pm 1	1 \pm 1	5 (<0.001)
Forskolin (basolateral)	26 \pm 7	24 \pm 5	5 (NS)
UTP (basolateral)	23 \pm 3	2 \pm 1	5 (<0.001)
Forskolin (basolateral)	50 \pm 10	25 \pm 3	5 (<0.05)
Forskolin (no UTP)	21 \pm 3	22 \pm 3	5 (NS)

The increase in I_{sc} (expressed in $\mu\text{A cm}^{-2}$) is calculated as the difference between basal current and peak current obtained within 15 min of addition of UTP (100 μ M) or forskolin (10 μ M).

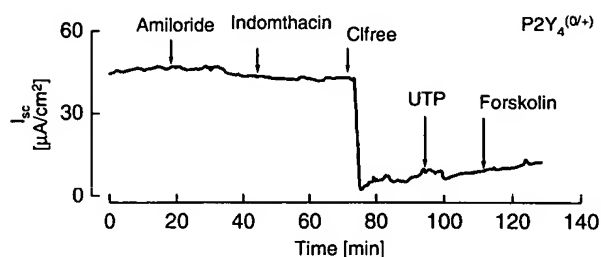


Figure 3 Effect of replacement by a chloride-free medium on the stimulation of I_{sc} by basolateral UTP and forskolin in the colon. In the chloride-free medium, chloride was replaced by isoosmotic gluconate. Amiloride and indomethacin were added as in Figure 2.

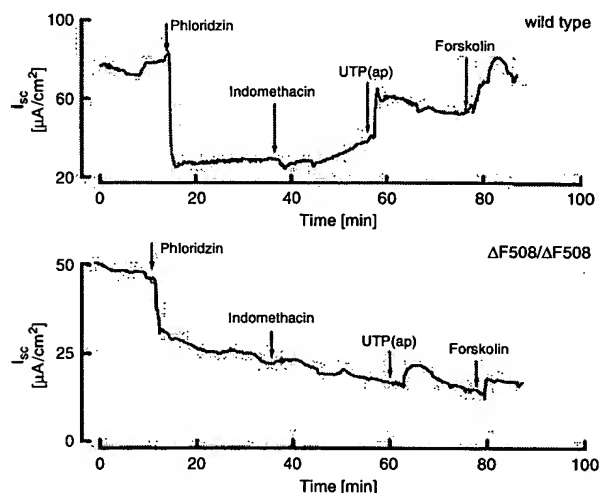


Figure 4 Stimulation of Cl^- secretion by apical UTP in the jejunum of control mice and mice homozygous for the ΔF508 mutation. Sodium–glucose cotransport activity was inhibited by adding 1 mM phloridzin to the apical solution. Indomethacin (100 μM) was added to both sides. UTP (100 μM) was added to the apical bath and forskolin (10 μM) to the basolateral one.

response to forskolin and that potentiation was abolished in $\text{P2Y}_4^{0/0}$ mice (Table 1). In a chloride-free medium, the resting value of the I_{sc} was greatly reduced and the response to apical UTP as well as basolateral forskolin was abolished (Figure 3).

Jejunal chloride secretion in ΔF508 mice

In the jejunum of mice homozygous for the ΔF508 mutation of CFTR, an increase in I_{sc} in response to apical UTP was present, though diminished as compared to wild-type littermates (Figure 4). It was $15 \pm 2 \mu\text{A cm}^{-2}$ as compared to $31 \pm 6 \mu\text{A cm}^{-2}$ (mean \pm s.e.m. of five animals). The response to forskolin was also decreased in $\Delta\text{F508}/\Delta\text{F508}$ as compared to wild-type littermates: $19 \mu\text{A cm}^{-2}$ versus $45 \mu\text{A cm}^{-2}$ (mean \pm s.e.m. of five animals).

Discussion

We have recently developed a new method to compute the actual values of the I_{sc} across leaky epithelia using impedance

analysis to estimate the transepithelial resistance as well as the resistance of the bathing solution (Ghanem *et al.*, 2005). Although such method may appear complicated, it should be emphasized that it is the only way to correct the recorded I_{sc} . The currents are indeed underestimated by the resistance of the bathing solution in series with the epithelium. For leaky epithelia, such as in jejunum and colon, this correction is important because the transepithelial resistance is in the same range as the solution resistance. Using this method, we demonstrate here the occurrence of chloride secretion across native murine colonic epithelium, a phenomenon so far observed only in cultured colonic epithelia (Kunzelmann & Mall, 2002). Furthermore, thanks to this method, we delineate the respective roles of P2Y₄ and P2Y₂ receptors in the chloride-secretory response to apical and basolateral UTP in jejunum and colon.

We have previously demonstrated that the chloride-secretory response to apical UTP is mediated entirely by the P2Y₄ receptor in the murine jejunum (Robaye *et al.*, 2003). We now show that the situation is different at the basolateral side, where both P2Y₄ and P2Y₂ receptors appear to play a role. There are precedents for such asymmetry in the literature (Dubyak, 2003). Differences between the responses to apical and basal UTP have been noticed previously in human colonic cells (Smitham & Barrett, 2001). It was recently shown that P2Y₂, P2Y₆ and P2Y₁₁ receptors are present on the luminal membrane of human nasal epithelial cells, whereas only P2Y₂ receptors are found on the basolateral membrane (Kim *et al.*, 2004). This asymmetry is also consistent with the involvement of different effector mechanisms activated by nucleotides: for instance, CFTR on the luminal side and $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter (NKCC) on the basolateral side (Köttgen *et al.*, 2003; Shin *et al.*, 2004). It was recently shown that, following transfection in Madin–Darby canine kidney cells, the P2Y₁, P2Y₁₁, P2Y₁₂ and P2Y₁₄ receptors reside at the basolateral membrane, whereas P2Y₂, P2Y₄ and P2Y₆ are expressed at the apical membrane (Wolff *et al.*, 2005). The authors suggested that the polarized targeting of P2Y receptor subtypes is not a function of the type of epithelial cells, and thus extrapolated that P2Y₂ and P2Y₄ are always apical. In contradiction to this oversimplistic rule, expression of both apical and basolateral P2Y₂ has been characterized in diverse epithelia (Homolya *et al.*, 1999). Our results also do not support this extrapolation, since we obtained evidence that in jejunum and colon functional P2Y₄ receptors are expressed in both basolateral and apical membranes, whereas in jejunum functional P2Y₂ receptors are only present on the basolateral side.

We have observed that UTP increases I_{sc} also in the colon: abolition of that response in chloride-free medium indicates that UTP stimulates chloride-secretion. A chloride-secretory response to ATP/UTP has been reported previously in human colonic cell lines, Caco-2 (Inoue *et al.*, 1997) and T84 (Smitham & Barrett, 2001), but not in the native human or murine colonic mucosa (Kunzelmann & Mall, 2002; Leipziger, 2003). The response to both apical and basolateral UTP (100 μM) was abolished in $\text{P2Y}_4^{0/0}$ and maintained in $\text{P2Y}_2^{-/-}$ mice. The potency of UTP is similar at recombinant murine P2Y₂ and P2Y₄ receptors, with EC_{50} below 1 μM , and 100 μM UTP produces a maximal effect on I_{sc} in the trachea (where P2Y₂ is expressed) and an almost maximal effect in the jejunum (where P2Y₄ is expressed) (Cressman *et al.*, 1999;

Lazarowski *et al.*, 2001). Therefore, although concentrations of UTP > 100 μ M were not tested, it can be safely concluded that the colonic responses to UTP involve exclusively the P2Y₄ subtype and not the P2Y₂ one.

Exposure to basolateral UTP prior to basolateral forskolin amplified the forskolin response in the colon, and this was totally dependent on the P2Y₄ receptor. It is well known that cAMP and [Ca²⁺]_i can regulate Cl⁻ secretion in a synergistic way: in colonic epithelial cells, cAMP directly activates apical CFTR, while Ca²⁺ stimulates basolateral SK4 K⁺ channels and thereby increases the driving force for Cl⁻ secretion (Kunzelmann & Mall, 2002). Such phenomenon did not occur when UTP was added at the luminal side. This is consistent with a partially compartmentalized signaling. Compartmentalized calcium signaling has been described in human nasal epithelial cells (Paradiso *et al.*, 1995; Shin *et al.*, 2004) and equine sweat gland cells (Wong & Ko, 2002). In Calu-3 cells, activation of apical A_{2B} receptors by adenosine induced cAMP signaling that remained restricted to the apical membrane (Huang *et al.*, 2001).

It was reported previously that the jejunal response to apical UTP was abolished in CFTR^{-/-} mice, in agreement with the concept that CFTR is the only chloride transporter present in the intestine apical membrane (Lazarowski *et al.*, 2001). In mice homozygous for the Δ F508 mutation of CFTR, a response to apical UTP was clearly detectable, though diminished, as compared to wild-type littermates. The response of the Δ F508/ Δ F508 mice to forskolin was previously reported to be about 30% of that observed in control mice (Van Doornink *et al.*, 1995). These results are consistent with the observation of a low expression of Δ F508-CFTR in the murine intestine, where it can be stimulated by pharmacolo-

gical agents (Van Doornink *et al.*, 1995; French *et al.*, 1996; Steagall & Drumm, 1999). It would therefore be tempting to speculate that the P2Y₄ receptor could be a pharmacological target to treat the intestinal abnormalities in the large number of patients harbouring the Δ F508 mutation. However, in other Δ F508/ Δ F508 mice, chloride secretion in the jejunum was totally unresponsive to cAMP (Zeihner *et al.*, 1995). Moreover, there are discrepancies on the membrane expression of human Δ F508-CFTR in the literature (Kälin *et al.*, 1999; Bronsveld *et al.*, 2001). A very recent study, based on both immunocytochemistry and Ussing chambers measurements, failed to detect any expression of the protein and any functional response to carbachol in rectal biopsies (Mall *et al.*, 2004). Although it might be argued that rectum is not representative of jejunum and that the UTP signaling mechanisms may be partially different from those of carbachol, this report suggested that this therapeutic possibility is not realistic. On the other hand, the role of the P2Y₄ receptor in infectious diarrhea remains to be evaluated, especially in view of the recent reports showing that epithelial invasion by enteropathogenic bacteria induces the release of nucleotides (McNamara *et al.*, 2001; Crane *et al.*, 2002; Van Nhieu *et al.*, 2003).

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Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis

MR Knowles, LL Clarke, and RC Boucher

Abstract

BACKGROUND. Cystic fibrosis is characterized by abnormal electrolyte transport across the epithelia of the airways. In particular, there is excessive sodium absorption and deficient chloride secretion. Drugs that block excessive sodium absorption may provide clinical benefit in cystic fibrosis, but there are no available therapeutic agents to improve chloride secretion. In vitro studies in cultured human-airway epithelia indicate that triphosphate nucleotides (ATP and UTP) induce chloride secretion through apical-membrane purinergic receptors.

METHODS. We tested the ability of nucleotides to induce chloride secretion in vivo in 9 normal subjects and 12 patients with cystic fibrosis by measuring responses of nasal transepithelial potential difference (PD) to superfusion of nucleotides. Changes in transepithelial bioelectric properties and the permeability of the apical membrane to chloride in response to extracellular (apical) UTP were determined with ion-selective microelectrodes in cultured nasal epithelia.

RESULTS. ATP and UTP induced chloride secretion in vivo in both groups. At their maximal effective concentrations of 10^{-4} M, ATP and UTP were more effective

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chloride secretagogues in the patients with cystic fibrosis (mean $[+/- \text{SE}]$ change in PD, $-19.8 \pm 1.4 \text{ mV}$ and $-15.0 \pm 1.7 \text{ mV}$, respectively) than in the normal subjects ($-6.9 \pm 0.6 \text{ mV}$ and $-8.1 \pm 0.9 \text{ mV}$, respectively). Microelectrode studies established that extracellular UTP stimulated a larger increase in PD and chloride secretory current in epithelial cells from patients with cystic fibrosis than in cells from normal subjects, by actions localized to the apical membrane. **CONCLUSIONS.** Extracellular nucleotides are effective in vivo chloride secretagogues in the nasal epithelia of patients with cystic fibrosis. The equipotency of ATP and UTP suggests that the effect is mediated by P2 nucleotide receptors. Selected nucleotides, such as UTP or nucleotide analogues, should be investigated as therapeutic agents for lung disease in cystic fibrosis.

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